Structural Investigations of the Polysaccharides of the Chlorophyceae

by

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ABSTRACT OF THESIS

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Part One Starch-Type Polysaccharides of the Green Seaweeds, Cladophora rupestris, Ulva lactuca and Chaetomorpha capillaris

Starch-type glucans have been separated from the hot water extracts of the green seaweeds, Cladophora rupestris, Ulva lactuca and Chaetomorpha capillaris. These glucans have been fractionated into amylose and amylopectin components, apart from Chaetomorpha glucan which, as a result of more drastic extraction, contained no amylose. The high positive rotation of each of the amyloses, their ready retrogradation from aqueous solution, their reduction of periodate and their β-amylolysis values were all in keeping with a linear structure of α-1,4- linked glucose units. They had slightly lower iodine binding capacties than potato amylose, and this feature, together with their low viscosities, indicates that they are much smaller molecules (DP ca. 300-600) than potato amylose (DP ca. 2000). The algal amylopectins, apart from their low viscosities, were very similar to potato amylopectin. Their rotations, properties of the iodine complexes, and α- and β-limits were the same as potato amylopectin, and like the latter, they reduced ca. 1 mole of periodate for every anhydroglucose unit.

Part Two The Water-Soluble Sulphated Polysaccharides of the Green Seaweeds, Cladophora rupestris, Chaetomorpha linum and Chaetomorpha capillaris.

The starch-free, hot water soluble polysaccharides of Cladophora rupestris, Chaetomorpha linum and Chaetomorpha capillaris have been studied. Each gave rise to galactose, arabinose, xylose and minor quantities of glucose and rhamnose on acid hydrolysis, had positive rotations ($[\alpha]_D$ +53- +75°), and also contained ester sulphate (12.3%-15.5%), ash (8.1-9.3%) and protein (ca. 19-25.6%). The sulphated polymer from Cladophora was partially purified by chromatography on DEAE-cellulose. The product had $[\alpha]_D$ +66°, SO_3(13.3%), protein (16.9%) and contained relatively increased proportions of xylose.

Treatment of the Cladophora polymer with methanolic hydrogen chloride gave a degraded polymer containing 2.3% ester sulphate. Comparison of the relative proportions of sugars in the sulphated and desulphated polysaccharides, before and after periodate oxidation, indicated that the desulphation process had produced periodate susceptible arabinose units.

Treatment of the Cladophora polymer with alkali also removed ester sulphate (5.3%). Since this reaction produced a neglible quantity of 3,6-anhydrogalactose, it appeared that sulphate had been removed from sulphated hydroxyls, adjacent and trans to free hydroxyl groupings. This was confirmed by the action of sodium methoxide on the polysaccharide, when major quantities of 2-O-methyl-L-xylose were formed. This sugar could only have arisen from 3-sulphated L-arabinose units in the original polymer. The same effect was observed with the polysaccharides from the Chaetomorpha species.

P.T.O.

Use other side if necessary.

Galactose-6-sulphate and arabinose-3-sulphate were characterised from partial acid hydrolysates of Cladophora polysaccharide, and a third substance was tentatively identified as a 1,4- or 1,5- linked 3-sulphated L-arabinopyranosyl-L-arabinose. The same compounds were recognised qualitatively in acid hydrolysates of the Chaetomorpha polymers. Proof was also obtained from partial hydrolysis and acetolysis for the presence of 6-O-β-D-galactopyranosyl-D-galactose and 3-O-β-D-galactopyranosyl-D-galactose.

No unequivocal proof has been forwarded for the heterogeneity of the sulphated polymers, but the overall evidence indicates that the polysaccharides may be composed of families of polydisperse molecules, in which the various sugar units are inter-linked.

To my Wife and my Mother

Contents

General Introduction

The seaweeds are perhaps the best known members of the group of plants called the Algae. They are found in greatest abundance along the coasts, and differ from land plants in not possessing true roots, stems or leaves.

Because they are among the most ancient of plants, it is interesting to speculate that algae are, in some respects, precursors of the complex land plant system. A study and comparison of their structural materials may therefore provide some information on this point.

The seaweeds themselves fall naturally and conveniently into four main groups, each characterised by the predominant pigmentation. These groups are the Phaeophyceae (brown), Rhodophyceae (red), Chlorophyceae (green) and Cyanophyceae (blue-green). The members of the first two groups are most readily obtained in large quantities and yield products of commercial value. For these reasons, they have been subjected to the most extensive investigation.

In the past, seaweeds have been harvested and used widely as sources of ash (soda and potash), iodine and food for human and animal consumption. This last use is still common, particularly in eastern countries. Nowadays, the mucilaginous extracts from seaweeds are applied extensively in industries such as pharmaceuticals, food, textiles, paper and cosmetics, principally because of their viscosity and gelling properties. It is recognised that these mucilaginous seaweed extracts

consist chiefly of polysaccharide material.

There is much confusion about the recognition and classification of some seaweed species, particularly in the Chlorophyceae. In addition, it is known that the proportions of seaweed polysaccharides from the same source can undergo wide seasonal variations[1]. Thus, before an investigation is begun, one should have particular regard to classification, source and time of collection of the species.

It is often found, particularly from the Chlorophyceae, that extracts are heavily contaminated with nitrogen containing substances (10-30%), which are probably proteinaceous. Numerous procedures have been employed, with varying degrees of success, to effect removal of this protein. Generally these result in loss of polysaccharide material.

Seaweeds synthesise several types of polysaccharide. A tentative division of these into reserve, basic skeletal and intercellular polysaccharides is sometimes made, but functional distinctions are not always clear cut. For example, it has been suggested that alginic acid, a characteristic component of brown algae, may exist in different forms which possess different functions in the plant's metabolism[2]. Thus one type may act as a skeletal cell component, while another may exist as mucilaginous material between the cells, and their demarcation is not easy. For this reason, it is probably simpler to compile a classification on the basis of the chemical constituents of the polysaccharides.

The more important seaweed polysaccharides will therefore be briefly reviewed under the following headings.

I. Neutral Polysaccharides

- A. Reserve Polymers
- B. Skeletal Materials

II. Acidic Polysaccharides containing Uronic Acid

- A. Alginic Acid

III. Acidic Polysaccharides containing Ester Sulphate

- A. Fucoidin of the Phaeophyceae
- B. Galactans of the Rhodophyceae
- C. Polysaccharides of the Chlorophyceae containing Galactose and Arabinose

IV. Acidic Polysaccharides containing Ester Sulphate and Uronic Acid Residues

- A. Heteropolysaccharides of the Chlorophyceae

In this account, greatest emphasis will be placed on the sulphated polymers of the red and green algae. The former have been the subject of many investigations in various laboratories, while Part Two of this thesis is concerned with

structural studies on the sulphated polysaccharides of two genera of the Chlorophyceae of the type described under section III C. The characterisation of starch-type glucans isolated from three green seaweeds is described in Part One of this thesis, and it is desirable to consider the occurrence of this type of polysaccharide in green algae generally, in a later discussion together with the results obtained in the present investigation.

General Review

I. Neutral Polysaccharides

In the algae, neutral polysaccharides appear to occur chiefly as A. Reserve carbohydrate material, or B. as the basic skeletal component of the plant cell.

A. Reserve Polymers

Algal reserve polysaccharides are found within the plant cell, as polymers of D-glucose which are readily soluble in hot or cold water. In the brown weeds, this is exemplified by laminarin (page 5), a β-1,3-linked glucan. Red seaweeds usually contain a glucan which has properties similar to the amylopectin fraction of land-plant starches.(page 7). Species of green seaweed have also been shown to synthesise starch-type polysaccharides. These are very similar to land-plant starches

and can be fractionated into linear (amylose) and branched (amylopectin) components.[3]

(i) Laminarin

Laminarin is a characteristic component of brown seaweeds, particularly Laminaria species. Its occurrence, both as soluble and insoluble forms, was noted in early studies, but no great chemical differences between these were apparent.[4] The isolation of 3-O-β-D-glucopyranosyl-D-glucose[5] (laminaribiose fig.1), and the results of methylation and periodate oxidation led to the formulation of laminarin as chains of about twenty residues of β-1,3-linked D-glucose.

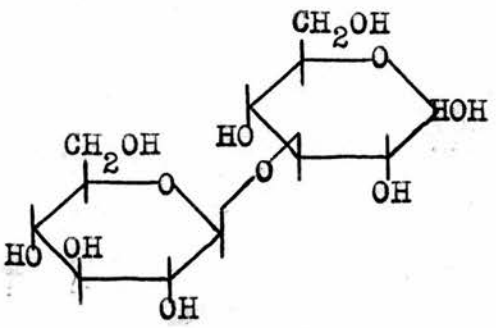
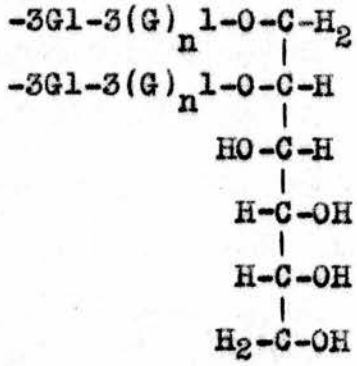
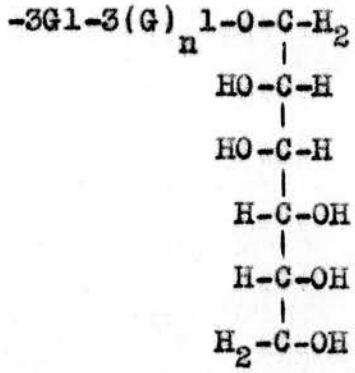


fig. 1 - laminaribiose

However, the conception of laminarin as a simple glucan was modified after the establishment of D-mannitol and β-1,6-linked D-glucose units as authentic structural features.[6] In particular, the isolation of 1-O-β-glucosyl-mannitol and 1-O-β-laminaribiosyl-mannitol, from partial hydrolysates of laminarin, led to the suggestion that D-mannitol constituted the terminal residue at the reducing end of some of the glucose chains.



Structure I



Structure II

fig. 2

Further work indicated that laminarin was a mixture of two molecules, one of which (laminarose) was reducing, i.e. terminated at the reducing end by a glucose unit, while the other (laminaritol) was non-reducing and terminated by a mannitol residue. By oxidation with bromine, the laminarose was converted to laminaric acid, which was then separated from the laminaritol by means of ion-exchange resins.[7]

Following the failure to detect ethylene glycol after a series of reactions involving oxidation with sodium metaperiodate, reduction with sodium borohydride and acid hydrolysis, it was suggested that, in laminaritol, one D-mannitol unit provided the terminal residue for two glucose chains.[7] (fig.2-structure I).

It has been shown by Manners and Annan, however, that such a series of reactions does, in fact, give rise to ethylene glycol. This, together with other evidence, has led these workers to conclude that structure II (fig.2) is the correct formulation.[8]

The presence of β-1,6-linked D-glucose residues in laminarin and its fractions has received support from periodate oxidation[9] and methylation studies.[10] The available evidence suggests that 1,6-linkages may be present in the polysaccharide, both within the linear chains and as inter-chain linkages. Moreover, recent work by Nisizawa has shown that samples of laminarin containing relatively high proportions of 1,6-linkages can be isolated from some species of brown seaweed.[11]

(ii) Floridean Starch

This polysaccharide is considered to be a reserve carbohydrate of the red seaweed Dilsea edulis, although it is probable that other red algae synthesise a comparable polysaccharide.

It was shown to be a starch-type glucan, in that it gave a red to violet stain with iodine,[12] was degraded to the extent of 50% with β-amylase and possessed a high positive rotation.[13] Although it was first suggested, on the basis of periodate oxidation studies, that the polymer contained about 40% of 1,3-linked residues,[14] subsequent investigations indicated that this was unlikely.[15,16] Nevertheless, the presence of a significant amount of nigerose in partial acid hydrolysates of floridean starch has led some workers to conclude that the polysaccharide does contain a small proportion of α-1,3-links as an integral part of its structure.[17] The presence of α-1,4- and α-1,6-D-glucosidic bonds was indicated from enzymic experiments, while an estimation of average chain length, from the amount of formic acid produced in periodate oxidation, was given as nine.[15]

Up to this time, the observations from different laboratories had tended to be at variance with one another, and there was some uncertainty whether the polymer more resembled amylopectin or glycogen. However, recent work on a sample of the polysaccharide purified by ultra centrifugation has provided some evidence that, in many respects, floridean starch is comparable with the amylopectin fraction of land plant starches.[18]

B. Skeletal Materials

Those polysaccharides which are considered to be skeletal substances are generally soluble in caustic alkali or zinc chloride solutions, after disintegration of the plant cells by a chlorite treatment. Studies by means of X-ray and electron microscope methods have indicated that a large number of algae contain a small proportion of cellulose as a skeletal material. However, the occurrence of skeletal polymers of D-xylose and D-mannose also appears to be widespread, especially in genera of the Chlorophyceae.

(i) Algal Cellulose

Cellulose is known to be the characteristic basic cell-wall component of most land plants. It consists essentially of long chains of β-1,4-linked D-glucose units.

The occurrence of a cellulosic polysaccharide in algal plants was first noted by Percival and Ross, in the brown seaweed species Laminaria and Fucus.[20] After exhaustive extraction with water and delignification with chlorine, the weed residues were treated with alkali. The polymer obtained by this means gave only glucose on acidic hydrolysis. It had a negative rotation, reduced one mole of periodate per anhydroglucose unit and gave rise to the octa-acetate of cellobiose (4-O-β-D-glucopyranosyl-D-glucose) on acetolysis. In addition, it possessed an X-ray diagram identical with that of cotton cellulose.

Few chemical investigations of algal celluloses have been reported since then, although the X-ray studies of Preston has

indicated the existence of cellulose in many species of red and green algae, as well as brown.[19] In accord with this, it has been shown, in this laboratory, that exhaustive extraction of the green weeds Cladophora and Chaetomorpha, provides residual materials which produce only glucose on acidic hydrolysis. It seems probable that this glucose arises from basic cellulose components of these weeds.

(ii) Algal Xylans

(a) From Rhodymenia palmata

The species of red seaweed R. palmata synthesises a xylan as a principal carbohydrate constituent. Unlike the other polysaccharides described in this section, this xylan is easily extracted from the crushed weed with dilute acid. It therefore seems possible that this polymer does not function as a structural material in the same sense as these other substances.

The results of periodate oxidation and methylation experiments suggested that the polysaccharide contained both 1,4- and 1,3- links in a ratio of 4 : 1, and that the average chain length was about twenty xylose units.[21,22] Evidence that the two types of linkage occur within the same molecule has been obtained from enzymic degradation experiments. Thus, digestion with preparations of a xylanase from sheeps' rumen, gave rise to oligosaccharides containing both 1,3- and 1,4-

linked D-xylose.[23] A recent examination has also indicated that few of the 1,3-linked units occur together in sequence, and that the molecule is rather larger than hitherto believed.[24]

(b) From Caulerpa filiformis[25]

Alkaline extraction of the chlorite treated, aqueous extracted weed, produced a polymer containing 95% D-xylose and 5% D-glucose residues. Further purification of this material removed the D-glucose residues to give a polysaccharide consisting solely of D-xylose. Methylation and periodate oxidation of the impure substance indicated that both the xylose and the glucose units were 1,3-linked, while from the negative rotation, it was inferred that the glycosidic bonds possessed the β-configuration. This was confirmed when partial acid hydrolysis experiments provided a series of β-1,3-linked D-xylose containing oligosaccharides. In contrast to the polymer from R. palmata (page 9), no evidence was obtained for the presence of 1,4-linked D-xylose units.

In addition to Caulerpa species, it has been reported, on the basis of acid hydrolysis and periodate oxidation experiments, that β-1,3-linked xylans form the basic structural component of the following genera of algae :- Bryopsis, Halimedia, Chlorodesmis and probably Udotea and Pseudodichotomosiphon.[26] It is interesting that the xylans from these genera were also intimately associated with 1,3- linked glucose units. (cf. Caulerpa filiformis).

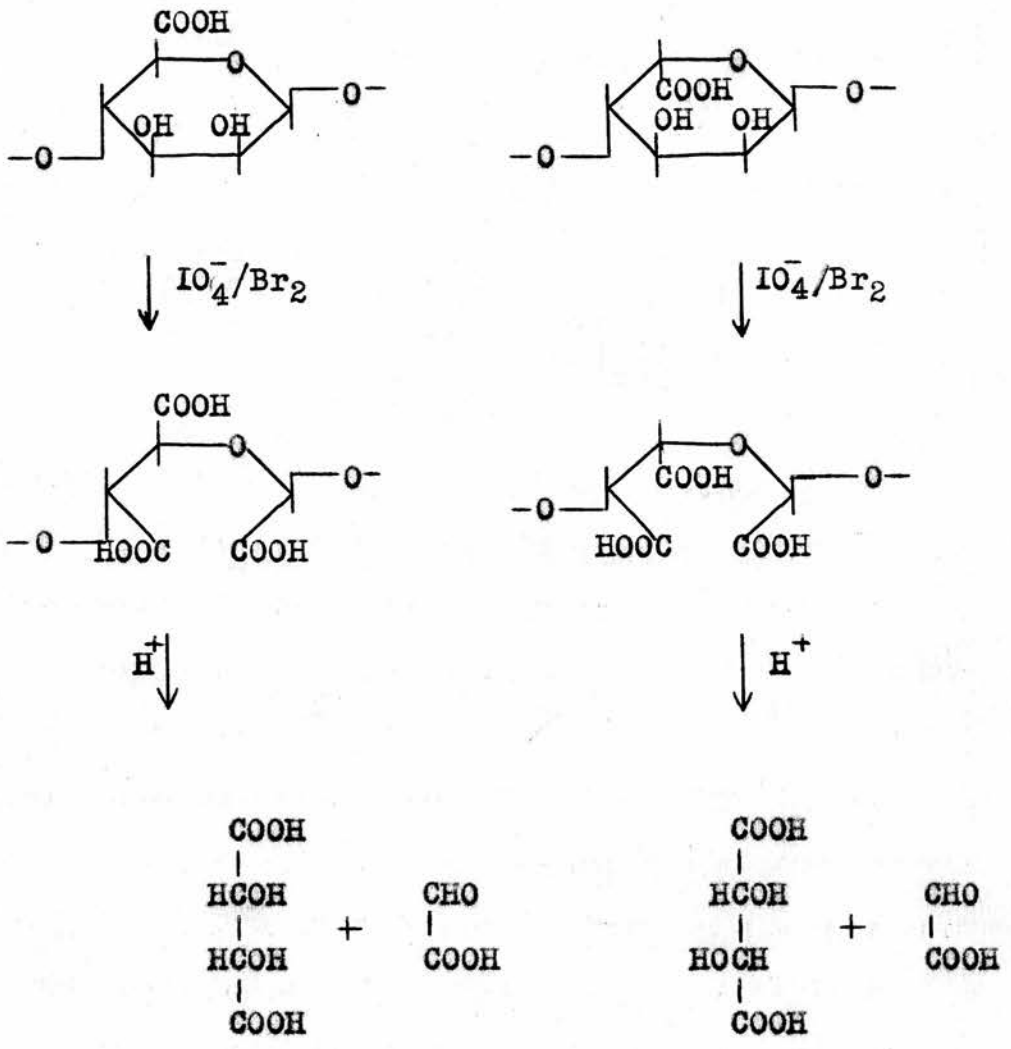
(iii) Algal Mannans

(a) From Porphyra umbilicalis[27]

After exhaustive extraction of the red seaweed P. umbilicalis with dilute acid, treatment with hot concentrated sodium hydroxide produced a polysaccharide solution, rich in D-mannose residues. This method of isolation suggests its function as skeletal polysaccharide material. The mannan was purified by the regeneration of the copper complex, when it was shown to consist largely of β-1,4- linked D-mannose units, with a small degree of branching.

(b) From Codium fragile[28]

A polymer containing D-mannose and a small amount of D-glucose (ca. 5%) was soluble in hot caustic alkali, following chlorite treatment of the residue remaining after exhaustive aqueous extraction of the green weed C. fragile. It had $[\alpha]_D$ -41°, after purification via its copper complex. Methylation and periodate oxidation established its structure as essentially a linear chain of 1,4- linked D-mannose units. In addition, the products of partial enzymic degradation included β-1,4-linked mannobiose and mannotriose, both of which were obtained in crystalline form. Evidence was subsequently obtained that glucose and mannose existed in the same molecule, when a small amount of a syrupy mannosyl-glucose disaccharide was isolated from a partial acetolysate of the polysaccharide.



D-erythraric acid L-threaric acid

fig. 3

Preliminary evidence has also been put forward for the occurrence of β-1,4- linked mannans as skeletal materials in the algal species Derbesia, Acetabularia and Halicoryne, as well as Codium. In contrast to the mannan obtained from C. fragile in this laboratory, these polymers apparently did not contain minor quantities of glucose.[26]

II. Acidic Polysaccharides containing Uronic Acid

A. Alginic Acid

Alginic acid is known to be a principal component of many species of brown seaweed. It is a polyuronide in that it appears to consist wholly of residues of uronic acid.[29] The presence of D-mannuronic acid was suggested early on[30] and subsequent evidence indicated that it was β-1,4- linked.[31] However, by virtue of improved chromatographic techniques, Fischer and Dörfel showed that L-guluronic acid also occurred as a main constituent.[32] The authenticity of L-guluronic acid as a structural feature was confirmed by other workers, and in addition, evidence that both the D-mannuronic and L-guluronic acids were 1,4- linked was presented.[33] Treatment of alginic acid with sodium metaperiodate, bromine and finally mineral acid gave a mixture containing both meso-tartaric (D-erythraric) and L(+)-tartaric (L-threaric) acids. (fig. 3). The optically active isomer could have arisen only from residues of 1,4-linked L-guluronic acid in the polysaccharide.

It has also been suggested that some of the acidic units exist as 3,6-lactones in the polymer, and this may explain the relative immunity of alginic acid to attack by periodate.

Although evidence has been put forward for the existence of polymannuronic and polyguluronic acid as separate species, recent work, involving partial hydrolysis of a reduced and esterified sample of alginic acid, has led to the isolation of a crystalline D-mannosyl-L-gulose disaccharide.[34] This indicates that at least some of these units occur together in one molecule.

III. Acidic Polysaccharides containing Ester Sulphate

The most characteristic group of seaweed polymers are the sulphated polysaccharides. Sulphated sugar residues have never been found in land plants, although they constitute a large proportion of some animal mucopolysaccharides. The algal polymers often contain more than one type of sugar unit, some or all of which, carry the sulphuric acid half ester residues. They are generally soluble in cold or hot water.

It is thought that these sulphated polysaccharides occur chiefly between the plant cells and serve as a cushion, to protect the cells from buffeting, as well as providing an ion exchange barrier between the cell and its environment. They may also help in preventing desiccation of the plant on exposure at low tide. For example, species of Ulva harvested from deep water yielded a polysaccharide of lower sulphate content than a sample gathered from the tidal zone.

It is probably true to say that these sulphated polymers submit least readily to a chemical investigation. Methylation is notoriously difficult and is generally incomplete, while attempts to simplify the molecules by desulphation may lead to extensive degradation.

It is perhaps relevant to mention here that these ester sulphates are readily hydrolysed by mineral acid at a rate similar to that of the glycosidic bonds. Some successful desulphations have been effected however, notably in instances where the glycosidic linkages have been stabilised by the presence of uronic acid groupings. (page 30). In these cases, treatment of the polysaccharides with methanolic hydrogen chloride at room temperature was found to be suitable for preferential cleavage of sulphate linkages with minimum rupture of the glycosidic bonds. In some polysaccharides the ester sulphate has proved more stable than the glycosidic linkages, and small proportions of some monosaccharide sulphates have been isolated from acid hydrolysates. These will be detailed in discussions of the particular polysaccharides.

The ester sulphate groups are exceedingly stable to alkali, except in certain circumstances.[35] For example, when a sulphate group exists in a trans position to a free, contiguous hydroxyl group, it is susceptible to alkaline hydrolysis in the following manner. Ionisation of the adjacent unsubstituted hydroxyl takes place and this is followed by the attack of this anionic species at the backside of the carbon atom bearing the sulphate residue. The sulphate grouping is expelled and a new bond is

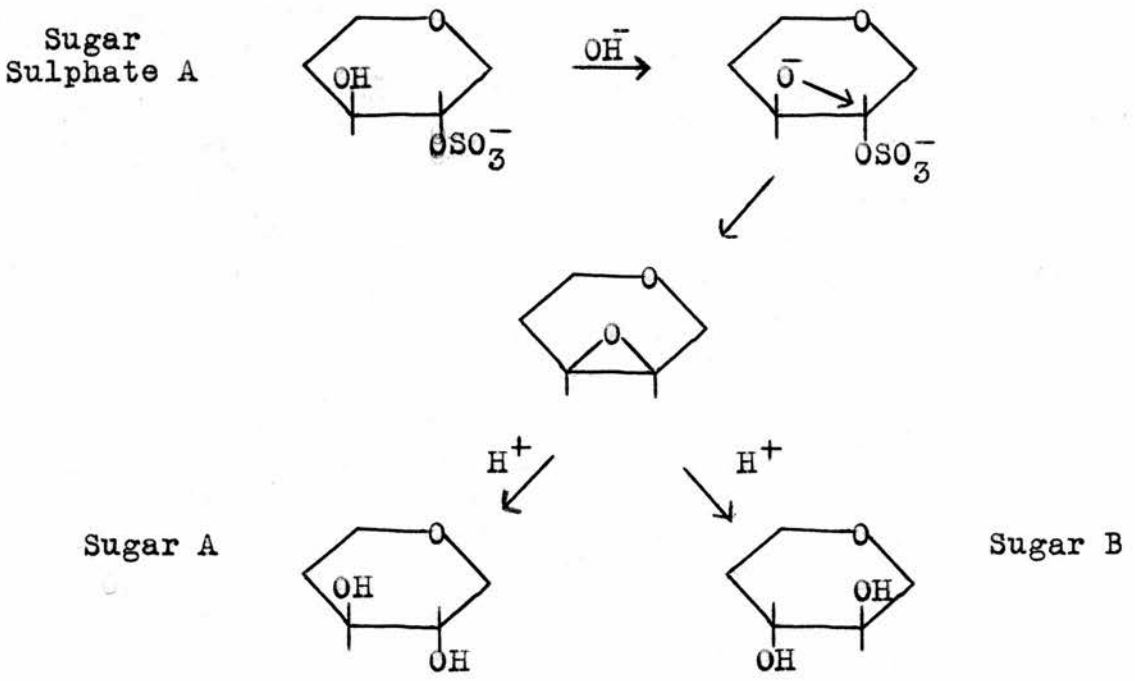
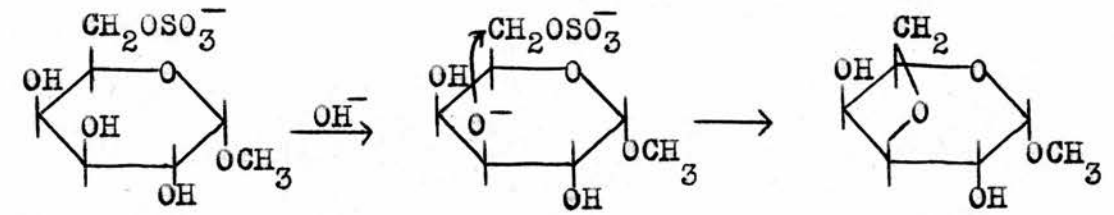


fig. 4



∝-methyl-galactose-6-sulphate

∝-methyl-3,6-anhydro-galactoside

fig. 5

made, with Walden inversion at the site of the original sulphate group, to form an epoxide ring. Hydrolysis of the epoxide ring may then take place in the presence of acid or alkali, with the formation of two products; the desulphated original sugar and a new one as shown. (fig. 4).

Furthermore, in the presence of alkali, hexoses which have a sulphate residue at C_6 and a suitably disposed hydroxyl at C_3, may undergo smooth conversion to the corresponding 3,6-anhydro derivative with concomitant loss of sulphate. (fig. 5).

A. Fucoidin of the Phaeophyceae

The sulphated polysaccharide characteristic of brown algae is known as fucoidin. It consists of residues of L-fucose (6-deoxy-L-galactose - 56.7%) and a high content of ester sulphate (38.3%). Other components such as D-galactose, D-xylose and uronic acid are thought to be impurities.[36]

Hydrolysis of methylated fucoidin produced a mixture of 2,3 di-O-methyl-L-fucose (20%), 3-O-methyl-L-fucose (60%) and L-fucose (20%).[37] The majority of sulphate residues were stable to alkali and this suggested that these were located on C_4. The methylation results were accordingly interpreted as being indicative of 1,2- linked L-fucose residues in the original polymer. This was confirmed when reduction of a partial acetolysis mixture of fucoidin gave rise to 2-O-α-L-fucopyranosyl-L-fucitol.[38] (fig. 6).

To account for the presence of free L-fucose among the

methylated products, it was suggested that some of the L-fucose might exist as 3,4-disulphated units or as multiple branch points. Similarly, 2,3-di-O-methyl-L-fucose could have arisen from 1,4- linked unsulphated units. It has been further suggested that 2,3-di-O-methyl-L-fucose could have arisen from 1,4- linked units carrying sulphate residues on either C_2 or C_3, since these would probably be removed during the methylation procedure. In support of this, it is known that 10% of the ester sulphate in fucoidin is alkali labile.[37]

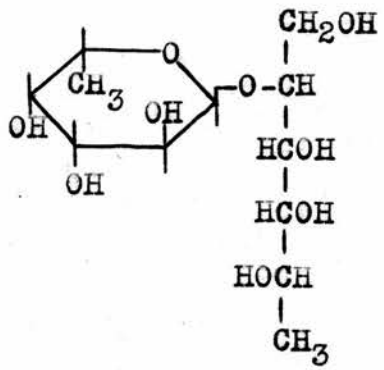


fig. 6 - 2-O-α-L-fucopyranosyl-L-fucitol

Recent acetolysis experiments have provided additional evidence for linkages other than 1,2- in fucoidin, following the isolation of 4-O-α-L-fucopyranosyl-L-fucose.[39]

B. Galactans of the Rhodophyceae

In many ways, the sulphated polysaccharides of the Rhodophyceae, like the hemicelluloses of higher plants, may be regarded as a family of closely related polysaccharides. Their structures are based on galactose, 3,6-anhydrogalactose and in some cases 6-O-methyl-D-galactose. Both galactose and 3,6-anhydrogalactose have been shown to occur as the D- and L-isomers, but only galactose itself has been found as both forms in a single polysaccharide molecule.

The best known examples of this kind of polymer are (i) carrageenan, (ii) agar and (iii) porphyran. These three substances exhibit broad similarities in sugar constituents and type of glycosidic linkage. At the same time, however, they possess fundamental differences of structure such as to render each a unique polysaccharide.

(i) Carrageenan[40,41]

The name carrageenan was applied to the gel forming water-soluble polysaccharide extracted from species of red algae, especially Chondrus crispus and Gigartina stellata.

Early studies indicated a preponderance of 1,3- linked D-galactose residues carrying ester sulphate groupings on C_4.[42] These were thought to exist in conjunction with an unknown constituent and a certain amount of L-galactose. Small amounts of glucose and xylose, usually present, were considered to be impurities.

The fractionation of carrageenan into two principal components,[43] ν-fraction (40%) and λ-fraction (60%), and the recognition of 3,6-anhydro-D-galactose as a main constituent of κ-carrageenan, were major steps forward.[44,45] A specimen of ν-carrageenan, which contained D-galactose, 3,6-anhydro-D-galactose and ester sulphate in the molar proportions 6 : 5 : 7, respectively, was subjected to a partial mercaptolysis procedure. In this way, the diethyl dithioacetal of 4-O-β-D-galactopyranosyl-3,6-anhydro-D-galactose (fig.7) was isolated and characterised.[46]

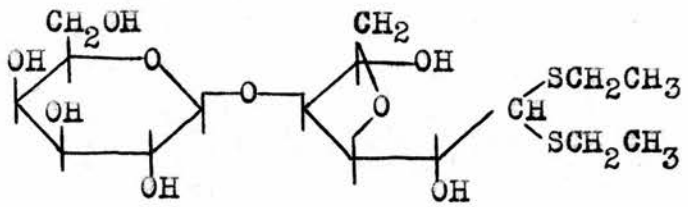


fig. 7

This feature, when considered along with the previous evidence, led to the suggestion that ν-carrageenan consisted primarily of chains of alternating residues of 1,3- linked D-galactose, some of which were sulphated on C_4, and 1,4- linked 3,6-anhydro-D-galactose. It was also suggested that a branch point occurred at C_6 of every tenth D-galactose unit, and that terminal non-reducing units were 3,4- or 3,6- disulphated.

Studies on λ-carrageenan suggested that it contained 1,3- linked D-galactose-4-sulphate units and very little 3,6-anhydro-D-galactose. Support for this was obtained by the isolation of a disaccharide, characterised as 3-O-α-D-galactopyranosyl-D-galactose after partial acetolysis of λ-carrageenan.[47] A crystalline trisaccharide was also tentatively identified as O-α-D-galactopyranosyl-(1-3)-O-α-D-galactopyranosyl-(1-3)-D-galactose.

The greater complexity of λ-carrageenan became apparent however, when Rees accumulated evidence for the existence of 1,4- linked D-galactose-2,6-disulphate residues to the extent of 40%.[48]

D-galactose-6-sulphate was isolated as a product of partial acid hydrolysis; treatment with alkali led to formation of 3,6-anhydrogalactose at the expense of 6-sulphate units; and from a partial mercaptolysate of the alkali treated polymer, a derivative of 4-O-β-D-galactopyranosyl-3,6-anhydro-D-galactose (fig. 7) was obtained, proving the existence of 1,4- linkages.

The alkali-modified carrageenan also resembled the naturally occurring κ-fractions from C. crispus and the related species, Hypnea specifera and Furcellaria fastigiata. It differed from these however, in containing 3,6-anhydrogalactosidic linkages which were less susceptible to acidic hydrolysis. It was suggested[50] that this additional stability was due to the presence of sulphate groupings on C_2 of the 3,6-anhydrogalactose residues, which had been formed from original D-galactose-2,6-disulphate units in the λ-carrageenan.

Comparative hydrolysis studies[51] of alkali-modified λ-carrageenan and the ϰ-fractions affirmed that the former did contain a labile ester sulphate component, in keeping with the presence of 2-sulphated D-galactose, as well as the more stable ester sulphate (presumably on C_4) present in the ϰ-fractions. Further proof that sulphate existed on C_2 was provided when a compound possessing the expected properties of 3,6-anhydro-D-galactitol-2-sulphate was isolated after a series of reactions designed to produce this species from the alkali-modified λ-carrageenan.

These studies have led Rees to suggest that the ϰ- and the λ-fractions of carrageenan possess a biological relationship, in which the 1,4- linked D-galactose-2,6-disulphated units in λ-carrageenan act as precursors of the 1,4- linked 3,6-anhydro-D-galactose units in ϰ-carrageenan.

It may also be mentioned that more recent investigations involving methylation of λ-carrageenan and a desulphated derivative, have provided additional confirmation that the molecule contains 40% of 1,4- linked D-galactose-2,6-disulphate units.[49]

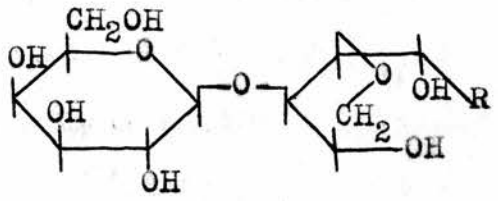
(ii) Agar[40,41]

Agar or agar-agar, like carrageenan, is a common gel forming constituent of some red seaweeds, for example, Gelidium and Gracilaria species. It differs from carrageenan in possessing the L-isomer of 3,6-anhydrogalactose and very little ester sulphate.

Although the chief components of agar are D-galactose and 3,6-anhydro-L-galactose, other minor constituents, mostly thought to be impurities, have been reported present. Hirase has recently shown that the agar-like polymer obtainable from [52] Ceramium species contains 6-O-methyl-D-galactose. In this respect, it resembles the sulphated polysaccharide from Porphyra umbilicalis. (page23).

Agar also resembles carrageenan in consisting of at least two main components, agarose (70%) and agaropectin (30%). [53] These may be separated as chloroform soluble and chloroform insoluble material respectively, after methylation or acetylation.

The isolation of 2,4,6-tri-O-methyl-D-galactose from methylated agar, by several workers, indicated that the D-galactose residues were 1,3- linked. [54,55,56] Evidence for the presence of 3,6-anhydro-L-galactose was obtained when further methylation of partly degraded methylated agar produced 2,4-di-O-methyl-3,6-anhydro-L-galactose. [57] The initial low sulphate content made it unlikely that the anhydride arose by an alkaline desulphation process during the methylation. (page 15). Confirmation of the pre-existence of 3,6-anhydro-L-galactose in agar, followed from the isolation of its dimethylacetal and its diethyl dithioacetal, [58] [59] after complete methanolysis and mercaptolysis of agar. Partial acid hydrolysis of agar resulted in the formation of a disaccharide - agarobiose. [60] (fig. 8a). This was characterised as 4-O-β-D-galactopyranosyl-3,6-anhydro-L-galactose, and so provided evidence that the 3,6-anhydro-L-galactose was 1,4- linked in the polysaccharide. In subsequent



(a) R -CHO

(b) R $-CH(OCH_3)_2$

(c) R $-CH(SC_2H_5)_2$

fig. 8 - agarobiose

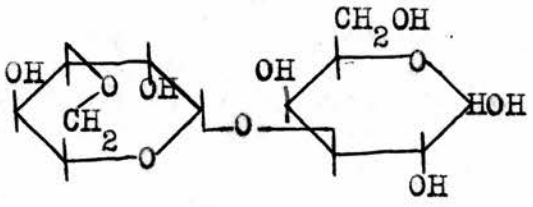


fig. 9 - neoagarobiose

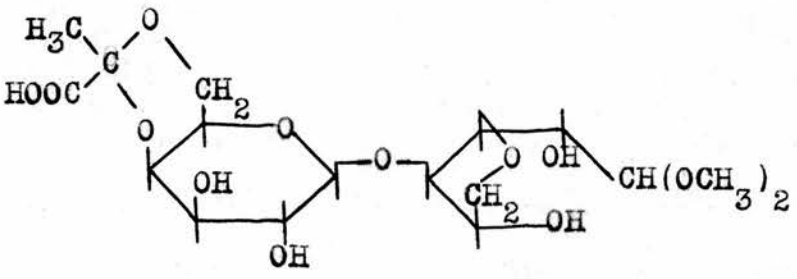


fig. 10

studies, the dimethylacetal (fig. 8b) and the diethyl dithioacetal (fig. 8c) of agarobiose were also isolated and identified.[61]

Fragmentation of the agarose molecule has also been effected with preparations of the bacterium Pseudomonas kyotoensis.[62] This produced a new disaccharide (neoagarobiose) and a tetrasaccharide (neoagarotetraose),[63] each isolated as crystalline substances. Neoagarobiose was shown to be 3-O-(3,6-anhydro-α-L-galactopyranosyl)-D-galactose. (fig. 9).

The identification of these two oligosaccharides gave more support to Araki's previous suggestion that agarose was composed of β-1,3- linked D-galactopyranose and α-1,4-linked 3,6-anhydro-L-galactopyranose residues, in alternating sequence as a long chain.[53] Although this general view has been confirmed by other workers,[64,65] and by more recent studies of Araki himself,[66] definite conclusions regarding the occurrence of sulphate and the nature of the end residue in the chain are not yet available.

Agaropectin, the minor component of agar, contains residues of pyruvic acid, glucuronic acid and a larger proportion of ester sulphate, in addition to agarobiose units.[41,67] The occurrence of pyruvic acid as an authentic structural feature has been verified by the isolation of a disaccharide, characterised as the dimethylacetal of 4,6-O-1-carboxyethylidene-β-D-galactopyranosyl-(1-4)-3,6-anhydro-L-galactose from a partial methanolysate of agar. (fig. 10). Further investigation is required, however, so that the situation of sulphate and uronic acid can be ascertained.

(iii) Porphyran

Porphyran is the name given to the sulphated polysaccharide obtained from the red seaweed Porphyra umbilicalis. Like agar and carrageenan, it is thought to be a mixture of related polysaccharides.

It has been shown that porphyran contains residues of D- and L-galactose, 3,6-anhydro-L-galactose and 6-O-methyl-D-galactose, in addition to ester sulphate.[68] Part of the ester sulphate has been located on C_6 of L-galactose units,[69] and some of the remainder is possibly situated on C_4 of 1,3-linked D-galactose residues. It has been demonstrated that enzymic preparations are able to effect the conversion of L-galactose-6-sulphate to 3,6-anhydro-L-galactose within the polysaccharide, and this transformation has been viewed as a normal reaction in the biosynthesis of the polymer.[70] The same transformation can be brought about by treatment with alkali.

Subsequent studies have resulted in the separation of the disaccharides 3-O-L-galactopyranosyl-D-galactose and 4-O-D-galactopyranosyl-L-galactose from an acid hydrolysate. Furthermore, in all samples of porphyran examined, 3,6-anhydro-L-galactose and L-galactose-6-sulphate comprised 50% of the sugar units, the remainder being D-galactose and 6-O-methyl-D-galactose.[71]

It therefore appears that this alga synthesises a DL-galactan, in which some of the 1,3- linked D-galactose units are methylated at C_6, while the ester sulphate occurs predominantly on C_6 of 1,4- linked L-galactose units which are

interconvertible with 3,6-anhydro-L-galactose.

It is convenient to mention here that infra red studies have also been helpful in locating ester sulphate groupings within some polysaccharides.

It is known that sulphate esters of D-galactose and D-glucose exhibit a general absorption band about 1240 cm.$^{-1}$ in the infra red. They also show specific bands which are considered to be characteristic of the type of ester sulphate, for example, at 820 cm.$^{-1}$ (sulphated primary hydroxyl), at 830 cm.$^{-1}$ (sulphated equatorial hydroxyl) and at 850 cm.$^{-1}$ (sulphated axial hydroxyl).[72]

Thus the spectra of carrageenan and porphyran support the presence of more than one type of ester sulphate in the polymers by showing two or more bands in the region 810 - 860 cm.$^{-1}$ This is in agreement with the results of other studies as described in the foregoing discussions.

These characteristic infra red absorptions have also been tentatively applied in assigning sulphate residues within polysaccharides of the Chlorophyceae, and these will be discussed more fully in the following sections.

C. Polysaccharides of the Chlorophyceae containing Galactose and Arabinose

All the genera of green seaweeds so far investigated have contained a water-soluble, sulphated polysaccharide as the major carbohydrate constituent, in addition to the neutral polymers described in other sections. None of these has been fractionated into simpler homopolysaccharides by standard techniques.

The studies to date have shown that the different genera of seaweeds fall into one of two groups, according to the type of polysaccharide synthesised. The first group includes (i) Cladophora rupestris, (ii) Caulerpa filiformis and (iii) Codium fragile. The polysaccharides of these genera of algae characteristically contain major proportions of D-galactose and L-arabinose, together with smaller amounts of D-xylose and L-rhamnose, but neglible quantities of uronic acid. Members of this group are discussed in this section.

The second main group includes the species Acrosiphonia, Enteromorpha and Ulva, whose polysaccharides are characterised by the presence of major proportions of D-glucuronic acid, L-rhamnose and D-xylose. Polymers of this class will be described in section IV.

(i) Cladophora rupestris[73]

The polysaccharide material from C. rupestris was among the first green seaweed polymers to be structurally investigated. The substance obtained after partial removal of proteinaceous materials had $[\alpha]_D$ +69°. It contained D-galactose, L-arabinose, D-xylose, L-rhamnose and D-glucose in molar proportions of 2.8 : 3.7 : 1.0 : 0.4 : 0.2, together with ester sulphate.(16.3% - SO_3). Application of numerous fractionation methods suggested that these sugars were part of a single molecule. In support of this, some tentative identifications of oligosaccharides containing arabinose, galactose and xylose were obtained.

Methylation of the polymer, though incomplete, indicated that some L-arabinose, D-galactose and L-rhamnose were involved in 1,3- linkages, while part of the D-xylose, at least, appeared to exist both as 1,4- linked units and as end residues. Periodate oxidation supported these conclusions since practically all of the xylose residues were eliminated, whereas some of the other monosaccharides remained intact in the oxidised polymer. The destruction of 66% of the galactose units also indicated that galactose linkages other than 1,3- were present in the polysaccharide.

No definite evidence concerning the distribution of sulphate groupings was obtained, although from their apparent stability to alkali, it was suggested that some of these might be situated on C_4 of galactose units.

A small proportion of a glucose rich fraction was

separated from the acetylated polysaccharide by extraction with chloroform. This was probably a laminarin-type polymer, (page 5), since it gave no stain with iodine and was immune to attack by periodate. In addition, a substance which was chromatographically and ionophoretically identical with laminaribiose, was obtained as a product of partial acidic hydrolysis.

Further advances in structural studies of the water-soluble polysaccharides of C. rupestris form part of the contents of this thesis. Part One describes a starch-type polymer synthesised by this weed, while Part Two presents some new evidence for structural details of the sulphated polysaccharide. Comparisons are also made with the water-soluble sulphated polymer obtained from Chaetomorpha species, which appears to be similar in many respects.

(ii) Caulerpa filiformis[74]

A polysaccharide, $[\alpha]_D$ +10.7°, containing D-galactose, D-xylose, and D-mannose in the molar proportions 5 : 1 : 2, was freed from a starch-type glucan by precipitation with cetyltrimethylammonium hydroxide in the presence of borate. The extract also contained ester sulphate (16.4% - SO_3), 3,6-anhydrohexose (ca. 1%) but was devoid of uronic acid.

A different sample of the same weed, from the same site, was found to contain a significant quantity of L-arabinose.

Reduction of periodate by the polymer was low, and a

proportion of all the different sugar units remained unattacked. On this basis, it was suggested that many of these were highly branched or 1,3- linked. Some of the sulphate was assigned to D-galactose units following the identification of a galactose-sulphate derivative from a partial acid hydrolysate.

(iii) Codium fragile[28]

The starch-free, hot water extract of C. fragile, after purification on diethylaminoethyl- (DEAE-) cellulose, contained ester sulphate (12.7% - SO_3) and anhydrohexose (0.74%). It had $[\alpha]_D$ +37.5°, and on acid hydrolysis gave rise to D-galactose and L-arabinose in molar proportions of 3.2 : 1.0, together with smaller amounts of D-xylose, L-rhamnose, D-glucose and D-mannose. Evidence for the presence of a high proportion of 1,3- linkages was obtained from periodate oxidation studies. This was confirmed by the isolation and characterisation of 3-O-β-D-galactopyranosyl-D-galactose and 3-O-β-L-arabinopyranosyl-L-arabinose from a partial acid hydrolysate of the polysaccharide. The situation of some of the ester sulphate on C_4 and C_6 of D-galactose units was confirmed by the separation and identification of both D-galactose-4-sulphate and D-galactose-6-sulphate from the products of partial acid hydrolysis. This was supported by the formation of 3,6-anhydrogalactose on treatment of the polysaccharide with alkali, and by the presence of bands at 850 $cm.^{-1}$ and 820 $cm.^{-1}$, in the infra red spectrum of the polymer. (page 24).

Molar Sugar Proportions	Acrosiphonia centralis [75]	Enteromorpha compressa [76]	Ulva lactuca [77]
Arabinose	-	-	-
Galactose	0.1	-	-
Xylose	1.6	2.7	3.5
Rhamnose	1.4	8.7	6.9
Glucose	1.0	1.0	1.0
Mannose	0.2	-	trace
Uronic acid (%)	19.3	18.3	14.1
Sulphate (SO_3-%)	6.5	13.6	15.9
$[\alpha]_D$	-31° *	-87°	-71°

table 1

* contains starch-type glucan

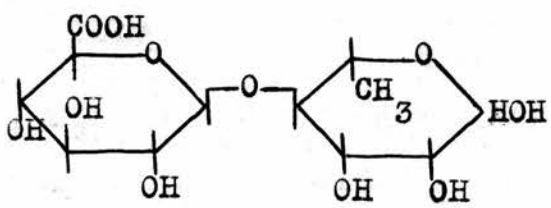


fig. 11 - 4-O-β-D-glucuronosyl-L-rhamnose

IV. Acidic Polysaccharides containing Ester Sulphate and Uronic Acid Residues

A. Heteropolysaccharides of the Chlorophyceae

This final section contains a brief account of the sulphated polysaccharides synthesised by the green seaweeds Acrosiphonia centralis (Spongomorpha arcta), Enteromorpha compressa and Ulva lactuca.

These substances are readily obtained, together with a small proportion of a starch-type polymer, by treatment of the decolourised weeds with hot water. In the cases of E. compressa and U. lactuca, the neutral glucans were removed by precipitation with iodine. An investigation of the starch-type glucan from U. lactuca is included in Part One of this thesis.

It has already been mentioned that this second group of green seaweed sulphated polymers differs distinctly from the first group, (III C; page 25), in that they contain a large proportion of D-glucuronic acid units. The accompanying table 1. shows to some extent how the sulphated polymers from these three genera of algae compare with each other.

It is interesting to note that although the seaweed A. centralis is botanically closely related to C. rupestris, these two seaweeds synthesise quite different sulphated polysaccharides.

In the absence of positive proof that these molecules are simply mixtures of homopolysaccharides, it appears that

they are best regarded as single heteropolysaccharides. Indeed it is probable that at least one heteropolymer exists, since all of these polysaccharides give rise to the same aldobiouronic acid, 4-O-β-D-glucuronosyl-L-rhamnose, (fig. 11, facing page 29), on acidic hydrolysis. The isolation of this disaccharide, in each case, emphasises the essential similarity of these polysaccharides. However, since they do show some divergences among themselves, for example, in sulphate content and in molar proportions of sugars, it is perhaps even better to consider them together as a family of closely related heteropolysaccharides. (cf. the galactan sulphates of the Rhodophyceae, page 17).

It has been suggested that each of these polysaccharides contains a proportion of 1,3- linkages or multiple branch points, since reduction of periodate is low and quantities of all the original monosaccharides remain intact in both the oxidised sulphated and oxidised desulphated polysaccharides.

The polymers of E. compressa and U. lactuca were the first green seaweed polysaccharides for which direct evidence for the location of sulphate residues was obtained, and so the remainder of this section will be devoted to a description of the methods used in these experiments.

Treatment of the starch free sulphated polysaccharide from E. compressa with methanolic hydrogen chloride effected removal of most of the ester sulphate, and left a residual

polymer (71%), which had apparently suffered little preferential degradation. The following constituents of the two substances were noted:-

		Glucose	Xylose	Rhamnose	SO_3	Uronic acid
Sulphated Polymer (%)	-87^{o}	5.7	15.0	45.0	13.6	18.3
Desulphated Polymer (%)	-88^{o}	9.8	15.0	51.0	0.6	23.5

The location of ester sulphate on some of the L-rhamnose units was deduced from a comparison of the periodate oxidised polymers. The desulphated polysaccharide reduced a larger fraction of periodate (0.68 mole) and the oxopolysaccharide contained a smaller proportion of unattacked L-rhamnose units (60%) than did the sulphated oxopolysaccharide (0.38 mole and 77%). It was pointed out that this sulphate could definitely be located on C_2 of L-rhamnose if certain assumptions were valid.

The sulphated polysaccharide showed an absorption band at 850 $cm.^{-1}$ in the infra red, which was not present in the spectrum of the desulphated material. It has already been mentioned that such an absorption is associated with the axially disposed sulphate group in D-galactose-4-sulphate. (page 28). If these absorption bands are applicable to all sugar sulphates, and if the L-rhamnose units exist in their most stable 1C conformation in the polysaccharide, then it follows that sulphate must be located on C_2 of these residues.

(the only hydroxyl which has an axial disposition in this conformation, fig. 12)

1C C1

fig. 12 - L-rhamnose

Similar methods have also been applied in studies of the sulphated polysaccharide from U. lactuca. As with the polysaccharide from E. compressa, it was shown that a partially desulphated polymer from U. lactuca (ca. 4% - SO_3) reduced a greater proportion of periodate (0.40 mole) than the original sulphated polysaccharide (0.20 mole), and that a smaller amount of uncleaved L-rhamnose units remained in the oxidised, desulphated material. The periodate oxidations were carried out in buffered solution at 2°C; conditions which have been shown to favour selective cleavage of cis-hydroxyl groups. The oxidation results were accordingly interpreted as providing additional evidence that L-rhamnose residues originally carried ester sulphate at C_2 or C_3, since these are the only sugar units, in this polymer, which contain the required cis-glycol system. Final evidence that sulphate was located on C_2 in L-rhamnose, was derived from an examination of the infra

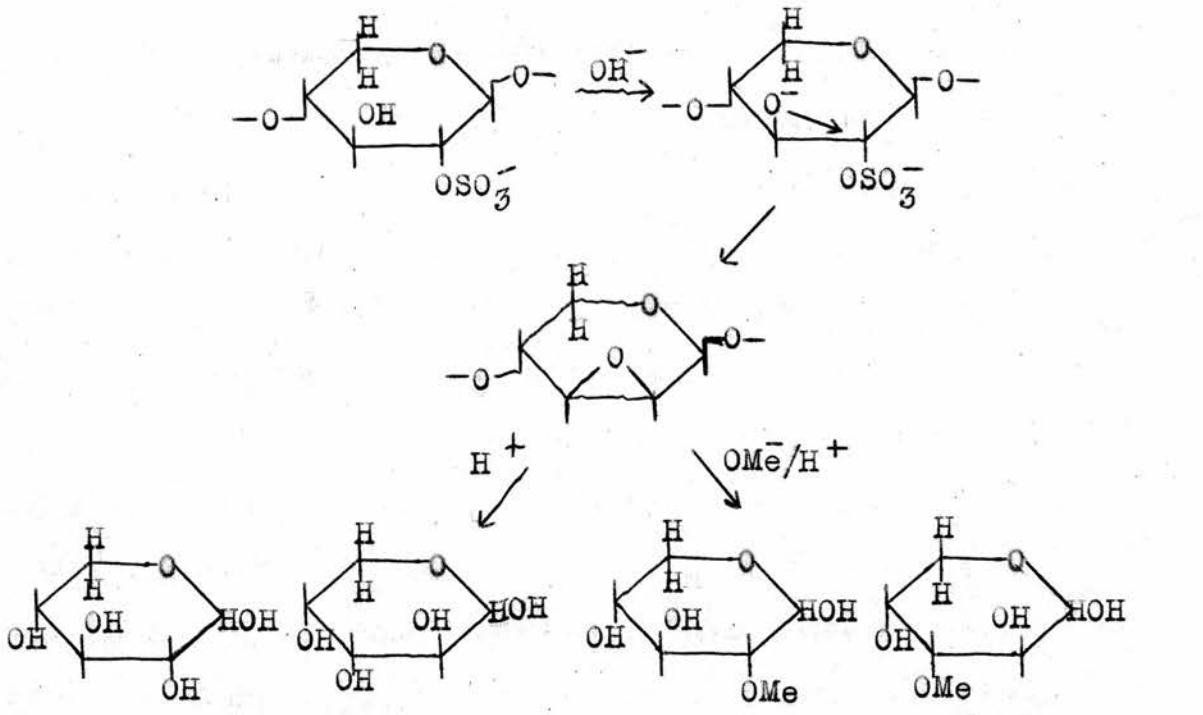


fig. 13a

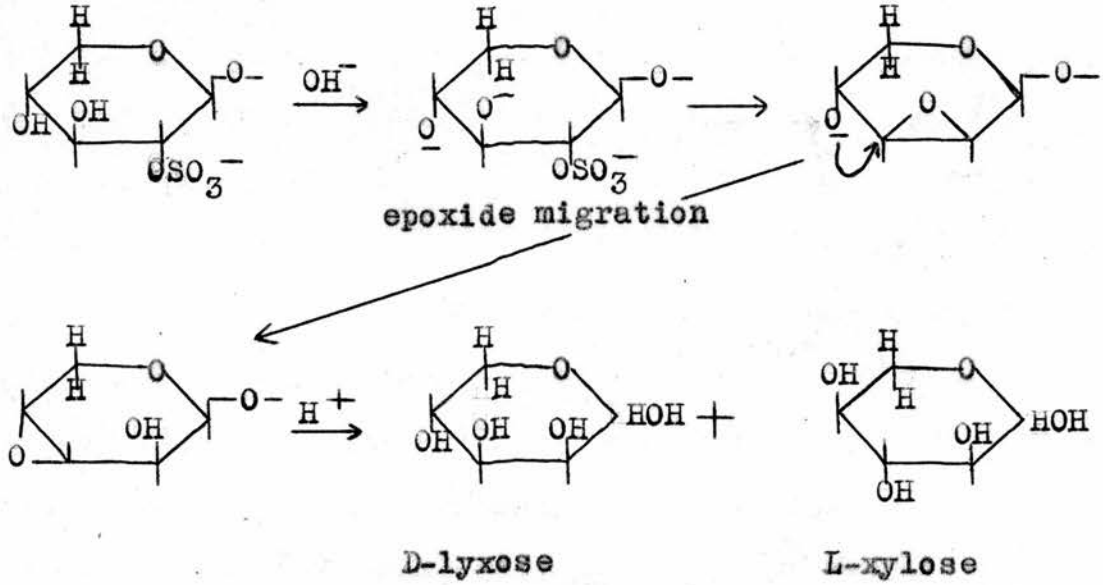


fig. 13b

red spectrum, in the same manner as described for the polymer from E. compressa.

Comparison of the periodate oxidised polysaccharides also indicated that some of the D-xylose units were sulphated, since the oxidised desulphated polymer contained a smaller proportion of D-xylose units (2.7 mole) than the corresponding sulphated material. (3.3 mole).

The presence of sulphated D-xylose was ascertained from the action of alkali on the sulphated polysaccharide. It was found that such a treatment resulted in the incorporation of new arabinose units within the polysaccharide, with concomitant loss of sulphate. The new sugar was isolated and characterised as D-arabinose. It was pointed out that this sugar could only have arisen from D-xylose units which were initially sulphated in the polysaccharide. The alkali-treated polysaccharide was refluxed in the presence of sodium methoxide, and from an acid hydrolysate of the recovered material, crystalline 2-O-methyl-D-xylose was isolated. This provided conclusive evidence that some of the D-xylose residues were sulphated on C_2. The reactions which are considered to bring about these transformations are depicted in the accompanying scheme. (fig. 13a).

As well as D-arabinose, a small quantity of tentatively identified D-lyxose was isolated from the acid hydrolysate of the alkali-treated polysaccharide. The formation of this sugar is most simply explained in terms of desulphation of D-xylose-2-sulphate terminal units, and subsequent migration

of the epoxide ring as shown in fig. 13b. The new ring would then undergo scission in the alkaline medium to give D-lyxose as one of the products.

It should perhaps be emphasised that all of these green seaweed polysaccharides discussed in sections III C and IV A, contained a residual quantity of nitrogenous substances. Since these could not be removed without seriously affecting the polysaccharide structures, the possibility always exists that these materials contain some carbohydrate-protein (or nucleotide) linkages.

General Methods of Investigation

Evaporations of carbohydrate solutions were carried out under reduced pressure at temperatures not greater than 40°C.

Isolation of Polysaccharides

Polysaccharide extracts were prepared by freeze-drying their aqueous solutions.

Drying

Samples were dried by storing overnight in a pistol-drier or vacuum oven, at 50 - 60°C, in the presence of phosphorus pentoxide.

Dialysis of polymer solutions was effected in cellophane bags, either against frequently changed distilled water, or running tap water, for at least 5 days. Toluene or chloroform was added to inhibit bacterial action.

Optical Rotations were determined in 1 dm. polarimeter tubes at room temperature in aqueous solutions, unless otherwise stated.

Ash Contents were estimated by ignition of samples (ca. 50 mg.) to constant weight in a platinum crucible.

Nitrogen Determinations[78] were obtained by the following micro-

Kjeldahl procedure. Polysaccharide, (30-50 mg.), catalyst (copper sulphate : sodium selenate : potassium sulphate - 20 : 1 : 80) and concentrated sulphuric acid (4 ml.) were digested together by heating overnight, with a small flame, in a Kjeldahl digestion flask. The contents were cooled, diluted with water and poured into a steam distillation apparatus. Several aqueous washings of the flask were also added to the distillation apparatus. Sodium hydroxide (15 ml.; 40%) was then added and the mixture was steam distilled. The evolved ammonia was absorbed in a boric acid trap (10 ml.; 4%), containing a mixed methylene blue/methyl red indicator (0.083 g. methylene blue and 0.125 g. methyl red in 100 ml. ethanol) and titrated with hydrochloric acid.

The product N% x 6.25 was used to obtain estimations of protein content.

Sulphate Estimations[79] were made by means of a modification of the procedure described by Jones and Letham.

Carbohydrate derivatives, containing ester sulphate (ca. 200 μg.), were digested overnight with analar concentrated nitric acid (0.25 ml.) at 140-150°C, in sealed tubes. Tubes were opened and taken to dryness overnight at 100-105°C, after the addition of sodium chloride. (1 mg.). The residue was taken up in water to give a concentration of 80-200 μg. sulphate. Aliquots were added to equal volumes of 4-chloro-4-aminodiphenyl (C.A.D.) reagent (0.19% in 0.1 N hydrochloric acid), containing a trace of cetavlon (cetyltrimethylammonium bromide). The

mixtures were left at room temperature for at least three hours, and after centrifugation, aliquots of the supernatants (0.1 ml.) were removed and diluted with hydrochloric acid (0.1 N; 10 ml.). The optical densities of the solutions were measured in a Unicam SP 500 spectrophotometer at 254 mμ. The values were subtracted from the corresponding reading found for an identically prepared sulphate free digest. The weights of sulphate were obtained from a calibration graph of the C.A.D. reagent with standard sulphate solutions. Sulphate contents are expressed as percent of SO_3.

Acid Hydrolysis

(i) Polysaccharide samples (10-30 mg.) were heated at 100°C with sulphuric acid (N; 2-5 ml.) for 5-7 hours. The cooled hydrolysates were neutralised with solid barium carbonate, filtered and deionised with Amberlite IR 120 (H) ion-exchange resin.

Alternatively, the hydrolysates were neutralised directly by shaking with a solution (5% in chloroform) of Amberlite LA-2 liquid anion exchanger, and the neutral aqueous solution washed with chloroform before concentrating to a syrup.

(ii) Small scale hydrolyses (200-1000 μg.) were carried out with hydrochloric acid (0.5N; 0.1 ml.) in sealed capillary tubes. The hydrolysates were examined chromatographically without prior neutralisation.

Estimation of Reducing Sugars

(i) Somogyi Cuprimetric Method[80]

Reducing sugars (0.4-5.0 mg.) were estimated by cuprimetric titration with the Somogyi reagent. Reduction equivalents (ml. 0.01 N sodium thiosulphate) were equated with weights of sugar by reference to a standard calibration curve.

(ii) Phenol-concentrated Sulphuric acid Reagent[81]

Carbohydrate solution (1 ml.), containing 20-80 μg. of sugar, was mixed with the phenol reagent (1 ml.; 5% w/v in water). Analar concentrated sulphuric acid (5 ml.) was added directly to the mixture from a fast flowing burette, whereupon an orange-yellow colour developed on gentle agitation. The optical density of the solution was read in a Unicam SP 500 spectrophotometer, at 490 mμ. (hexoses) and 480 mμ. (pentoses), or alternatively, in an EEL colourimeter with a green (623 mμ.) filter. A blank determination employing distilled water was prepared at the same time, and sugar concentrations were then calculated by reference to known solutions or standard graphs.

Degree of Polymerisation (DP)[82]

This was determined according to the Timell modification of the method of Peat, Whelan and Roberts.[83]

Three solutions were prepared as follows:-

(i) A blank containing water (0.5 ml.) and potassium borohydride solution (0.5 ml.;2%).

(ii) An aqueous sugar solution (0.5 ml.; containing 60-80 μg. as monosaccharide) and potassium borohydride solution (0.5 ml.; 2%).

(iii) A sulphuric acid (0.5 ml.; 2 N) solution containing the same weight of sugar as solution (ii), and potassium borohydride solution (0.5 ml.; 2%).

These mixtures were left at room temperature for 6-20 hours. Aqueous phenol (1 ml.; 5%) and concentrated sulphuric acid (5 ml.) were added and the mixtures shaken to develop the orange colour.

The degree of polymerisation (DP) of the carbohydrate substance is given by the relationship $\frac{Q}{Q-1}$, where Q is the ratio of the optical densities of the non-reduced (iii) and reduced (ii) solutions respectively.

Methylation[84] of small quantities of carbohydrate (0.5-2 mg.) was carried out according to the modified Kuhn procedure described by Bishop and Perila.

Carbohydrate in redistilled dimethylformamide (0.2 ml.) was cooled to 0°C before adding redistilled methyl iodide (0.2 ml.) and dry silver oxide (0.2 g.).

The mixture was shaken in a stoppered blackened flask for 2-3 hours, in an ice-bath, and then for 18-20 hours at room temperature. The product was filtered and the residue washed with chloroform. The filtrate plus washings were concentrated to dryness under high vacuum (0.1 mm.; 3-5 minutes) to remove dimethylformamide.

The dry residue was taken up in dry chloroform, filtered and concentrated to a syrup by aeration.

Methanolysis was carried out by refluxing sugars (0.5-10 mg.) with dry methanolic hydrogen chloride (3%; prepared by adding 5 ml. acetyl chloride to 100 ml. dry methanol) for 7-20 hours. The acidic solution was neutralised with dry siver carbonate, filtered and concentrated to a syrup.

Gas-Liquid Chromatography[85]

This was very kindly carried out by Dr. G. O. Aspinall on a Pye Argon Chromatograph in which the inert gas argon was employed as the mobile gas phase. The stationary liquid phase was supported on celite and was either :-

(i) Butanediolsuccinate polyester at an operating temperature of 175°C, or,

(ii) Polyphenol at an operating temperature of 200°C.

Retention times of the methyl glycosides of the monosaccharide methyl ethers were expressed relative to that of methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside.

Demethylation[86]

Methylated sugar (1-10 mg.) was added to dry dichloromethane (2 ml.) and cooled to -80°C. Boron trichloride (2 ml.), at -80°C, was added and the mixture kept at this temperature, under anhydrous conditions, for one hour. The mixture was then allowed to attain room temperature and left in a dry atmosphere

for 20 hours. The final solution was evaporated to dryness, with several additions of methanol, and concentrated to a syrup.

Periodate Oxidation[87]

This was normally carried out with sodium metaperiodate solution (0.015 molar) in the dark, at 0°C or at room temperature.

At intervals, the reduction of periodate was measured by removing an aliquot of the reaction mixture (0.100 ml.), and diluting this with water (25 ml.), before reading the optical density at 223 mμ. in a Unicam SP 500 spectrophotometer against a water blank.

The fraction of periodate reduced was then calculated, knowing the difference in absorbance between 0.015 molar sodium periodate and 0.015 molar sodium iodate, under the same conditions.

Ionophoresis of sulphated sugars was carried out on Whatman No. 1 or 3MM filter paper (2.5 x 15 cm.), in pyridine/acetic acid buffer (0.05 N; pH 6) with a potential of 300 volts, for 1.5 - 3 hours. Migration values (M_G) are expressed relative to the distance migrated by galactose-6-sulphate, after allowance for end osmosis migration.

Paper Chromatography

Qualitative and quantitative work was carried out on Whatman No. 1 filter paper. In preparative work, water washed Whatman 3MM filter sheets were used. The following notation was used to describe the mobility of sugars on paper chromatograms.

$$R_X = \frac{\text{Distance travelled by Sugar}}{\text{Distance travelled by SugarX}}$$

The following eluting mixtures were used:-

1. Ethyl acetate/Pyridine/Water 10/4/3
2. Ethyl acetate/Acetic acid/Formic acid/Water 18/3/1/4
3. Ethyl acetate/Pyridine/Water 8/2/1
4. Ethyl-methyl ketone half saturated with water containing one drop of concentrated ammonia.

Sugars were located by means of the following reagents:-

1. Aniline oxalate (reducing sugars only)

This was a saturated aqueous solution. Chromatograms were heated at 110°C for 5-10 minutes to develop the coloured spots; brown for hexoses, cherry-red for pentoses and yellow for deoxyhexoses.

2. p-Anisidine hydrochloride (reducing sugars only)

This was a 3% (w/v) solution of the reagent in water-saturated butanol. Colours were developed by heating at 105-110°C for 3-5 minutes.

3. Silver nitrate (reducing and non-reducing sugars)

Chromatograms were dipped in the silver nitrate reagent, (1 ml.saturated aqueous silver nitrate in 200 ml. acetone plus water to redissolve), and dried thoroughly in air. Sugars were revealed by spraying with alkaline ethanol (1 ml. 40% sodium hydroxide in 30 ml. ethanol). After drying in air, the papers were washed, first with sodium thiosulphate and then with water.

4. Bromocresol green

Acidic sugars were detected by spraying with an ethanolic solution (1%) made just alkaline with sodium hydroxide.

5. Triphenyltetrazolium hydroxide[88]

Papers were sprayed with the reagent, prepared by mixing equal volumes of aqueous triphenyltetrazolium chloride (2%) and sodium hydroxide (N), and kept in a moist atmosphere at 40°C for 10-20 minutes. The papers were then washed and and dried at room temperature. Sugars substituted at C_2 did not react with this reagent, whereas others produced a deep red colour.

6. Aniline/Diphenylamine/Phosphoric acid[89]

Two solutions were prepared as follows:-

I. Aniline (4 ml.) in acetone (100 ml.)

II. Diphenylamine (4 g.) in acetone (100 ml.)

Solutions I and II were mixed with orthophosphoric acid (20 ml.; 85%) and kept at 0°C. Chromatograms were dipped in the solution and warmed at 80°C for 3-5 minutes. Oligosaccharides which contained 1,4- glycosidic linkages produced bright blue colours, while others gave a greyish-green colouration.

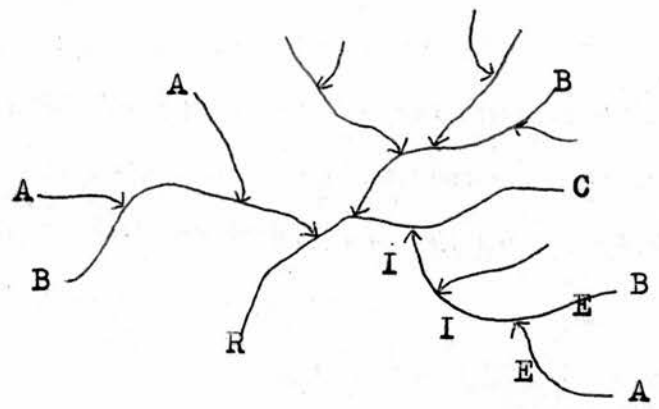
7. Toluidine blue

Sulphated carbohydrates were detected with toluidine blue (0.06% in 0.5% aqueous acetic acid). The stained papers were washed lightly in 2% aqueous acetic acid to remove excess dye, washed with water and dried in air.

Part One

The Starch-Type Polysaccharides of the Green Seaweeds

Cladophora rupestris, *Ulva lactuca* and *Chaetomorpha capillaris*



~ Chains of α-1,4- linked D-glucose residues.

A-chains are attached to the molecule, via the reducing group, by one α-1,6- link.

B-chains carry one or more A-chains, and are themselves attached to the molecule, via the reducing group, by one α-1,6- link.

C-chain carries the terminal reducing group (R) of the molecule.

⟶ Represents one α-1,6- link.

fig. 14

Introduction

Starch[90,91] is well known as the reserve polysaccharide of land plants. Starch-type polysaccharides have also been found in bacteria, protozoa and some algae.[92] It is recognised that starch usually occurs as a mixture of linear and branched polysaccharides, in proportions which can vary widely according to the source.

The linear component, amylose, is essentially built up from several thousand α-1,4- linked D-glucose units. Amylopectin, the branched component, also consists of chains of α-1,4- linked D-glucose residues, but these are much shorter (average length 20-25 glucose units), and are inter-joined to produce a multiply branched molecule. (fig. 14). The branches are known to be linked by way of α-1,6-D-glucosidic bonds.

The tree-like structure shown (fig. 14) was first suggested by Meyer.[93] In this representation three types of chain are distinguished as indicated. In addition, the portion of the chain between a terminal non-reducing unit and an outermost branch point is called an exterior chain (E). An interior chain (I) is the portion between two branch points.

Glycogen, the reserve polysaccharide of animal tissues, is of similar structure to amylopectin. However, it differs in possessing chains of shorter average length (9-14).[90]

These three types of polymer can often be distinguished quantitatively and qualitatively by the colour they give in

the presence of iodine. Amylose gives an intense blue, amylopectin gives a purple-red, while glycogen produces a brownish-red colouration.

The foregoing structures for amylose, amylopectin and glycogen have been firmly established by the results of classical chemical procedures such as methylation, periodate oxidation and graded acid hydrolysis. Nevertheless, much of the fine structure of glycogen and starch fractions has been elucidated by means of enzymic studies. Since some of these enzyme preparations were used in the present investigations, a short account of their action pattern is desirable.[90]

(i) <u>α-Amylases</u>

These enzymes are found in bacterial extracts, barley malt and mammalian pancreatic and salivary secretions. They effect a random hydrolysis of α-1,4-D-glucosidic bonds with the production of α-maltose.[94] Although they cannot hydrolyse α-1,6- links, they can circumvent them, and so degrade the inner chains of branched molecules. Linear substrates give maltose as the principal product, while branched substrates yield a proportion of higher oligosaccharides (α-limit dextrins) in addition to maltose.

(ii) <u>β-Amylases</u>

These enzymes are found in higher plants. They catalyse a step-wise hydrolysis of alternate bonds in a chain of α-1,4-linked D-glucose residues and produce β-maltose.[94] The degradation

proceeds from the non-reducing end of a chain and action is arrested only by the presence of structural variants, such as inter-chain linkages. Highly purified β-amylases degrade the exterior chains of amylopectin to within two or three residues from a branch point.[90,95] The products are maltose (50-60%), and a high molecular weight β-limit dextrin. Degradation of amylose usually produces 70-80% maltose and not ca. 100% as would be expected.[96] It has recently been demonstrated that the barriers to complete β-amylolysis in amylose are probably a small proportion of inter-chain α-1,6-D-glucosidic bonds, such as occur in amylopectin.[97] However, it is known that impure preparations of β-amylase which contain Z-enzyme can degrade amylose completely.[96] It is thought that this enzyme acts in a similar manner to α-amylase and catalyses a random hydrolysis of α-1,4- linked glucans.[98] In this way, the molecule is exposed to further scission by β-amylase. The concentration of Z-enzyme is normally sufficient to circumvent all of the inter-chain bonds in amylose, so that the molecule can subsequently be completely degraded to maltose by the β-amylase. On the other hand, branched substrates, such as amylopectin, contain such a high concentration of inter-chain linkages that only a small additional production of maltose is noticeable.

The action of Z-enzyme is inhibited at pH 3.6, but not at pH 4.6, while β-amylase is effective at both pH values. A comparison of the amounts of maltose produced from amylose and amylopectin by digestion with β-amylase at both pH values

can therefore provide a measure of distinction between the two types of molecule.

Experimental

Two samples of Cladophora rupestris were obtained by Dr. E. E. Percival.

(i) Collected from the shore at Dunbar, Scotland in October, 1954.

(ii) Collected from the same site in October, 1961.

Both of these samples gave rise to the same carbohydrate components, in the same proportions, on acidic hydrolysis.

The specimen of Ulva lactuca was gathered in Nova Scotia, in July, 1960, by Miss Constance Macfarlane.

The specimen of Chaetomorpha capillaris was collected at North Berwick, Scotland, in November, 1958, by Dr. E. E. Percival.

All samples of seaweed were rapidly dried in a current of air at room temperature before examination.

Expt. 1 Preliminary Treatment of C. rupestris Weed

The dried seaweed fronds were frozen with liquid nitrogen and ground up to a fine powder with a pestle and mortar. This material was steeped in n-butanol, half saturated with water (20 hours), filtered and treated with cold acetone until no further colour was removed. Portions of the decolourised weed were treated separately with water at different temperatures:-

(a) at room temperature

(b) at 60-70 ^{o}C

(c) at 95-100 ^{o}C

At intervals, aliquots of the mixtures were removed and clarified on the centrifuge. The supernatants were then tested with dilute iodine solution. No effect was observed with (a) or (b) after several days and hours respectively. On the other hand, after extraction for 20 minutes, the solution from (c) produced an intense blue colouration in the presence of iodine. This indicated that a starch-type polymer was being extracted in treatment (c), but not in (a) or (b).

Expt. 2 Large Scale Extraction of C. rupestris Weed

Decolourised weed (200 g.) was suspended in water (4 litres) and heated on a boiling water bath, with gentle stirring, in an atmosphere of nitrogen. After 6 hours, the extract gave a deep blue colour with iodine. Solid material was removed on the centrifuge, after extraction for 20 hours, and the dark brown supernatant was concentrated to a small volume, (500 ml.), whereupon it set to a stiff gel.

The formation of a starch-iodine complex was apparently inhibited in this gelatinous medium, since no blue colour could be produced with iodine. Dispersion of this gel was attempted by addition of ammonium carbonate solution (0.3%; 1 litre), and by means of ethylenediamine tetraacetic acid (EDTA), but neither of these procedures was successful. The gel was finally broken by addition of trichloroacetic acid to a concentration of 4% (w/v), followed by adjustment of the pH to 4 with sodium hydroxide. This mixture was left

overnight at 4°C, centrifuged and dialysed for one week. The non-diffusible solution was concentrated and freeze-dried to an off-white solid. (30 g.).

Since a hot-water solution of this solid gave only a pale pink colour with iodine, it seems probable that the starch had been severely degraded, either during the prolonged extraction , or in the ensuing acidic treatment.

Expt. 3 Improved Method for Extraction of Starch-Type Glucan

(a) Finely powdered, decolourised weed (30-40 g. batches) and water (500 ml.) were thoroughly saturated with nitrogen and the mixture heated to 90-95°C with continuous stirring and passage of nitrogen. After one hour, the mixture was cooled and the residual weed removed by centrifugation. The residue was washed with warm water and re-extracted as before. The extract and washings were concentrated to 200 ml. In this way, 100 g. of weed were extracted and treated according to the method of Steiner and Guthrie[99] as follows.

Aqueous sodium chloride (20%, w/v; 60 ml.), Celite (grade 545; 3 g.) and iodine solution (12% I_2 in 20% KI; 5 ml.) were added to the extract and shaken vigorously for 5 minutes in a stoppered flask.

Centrifugation yielded a black precipitate and a brown supernatant solution. The brown solution was dialysed free from inorganic substances and freeze-dried to give a starch-free sulphated polysaccharide (13.8 g.). This material was

kept apart from the trichloroacetic acid treated material obtained in Expt. 2., and both substances were used for the investigations described in Part Two of this thesis.

The black starch-iodine complex was immediately dispersed in sodium chloride solution (20%; 200 ml.) and stirred while sodium thiosulphate (0.5 N) was added dropwise to destroy the coloured complex. To the colourless solution were then added hydrochloric acid (N; 20 ml.), and a further volume of iodine solution.(10 ml.). The mixture was shaken vigorously as before, and the black complex removed and suspended in ethanol. (95%; 200 ml.). The suspension was stirred while the complex was once more destroyed by sodium thiosulphate. The ethanol concentration was reduced to 70% with water, after which the Celite-starch mixture was filtered off under suction and washed with ethanol. (70%; 500 ml.). This was made into a paste with water then plunged into water (100 ml.) at 90°C, in an atmosphere of nitrogen. After vigorous stirring for 15 minutes, the starch solution was obtained by filtration and dialysis, prior to freeze-drying.

The polysaccharide (0.7 g.) was obtained as an off-white solid. It gave an intense blue colour with iodine and produced glucose and a trace of arabinose on acidic hydrolysis. (chromatographic examination). It was contaminated with ash (6%) and protein (ca. 6%), and was only partially soluble in water and dilute alkali. The insolubility of the freeze-dried glucan impeded subsequent efforts at fractionation so that poor quality materials only, could be isolated in low yield.

The high ash content of the polymer probably arose from contaminating Celite.

(b) The above procedure was therefore repeated with the following modifications:-

(i) The amount of Celite was reduced to 1 g.

(ii) The Celite-starch mixture was subjected to high-speed centrifugation (12,000 r.p.m.) to remove finely suspended Celite particles.

The derived starch gave a clear colourless solution. It was contaminated with ash (2%) and protein (5%).[78] It had $[\alpha]_D^{80}+193^\circ$ (c. 0.6, found by cuprimetric titration of a hydrolysed, neutralised aliquot), and gave a blue colour with iodine. The total recovery was 1.0 g. from 100 g. of the dry seaweed.

Expt. 4 Fractionation of Cladophora Glucan into Amylose and Amylopectin Components

A quantity of the starch solution obtained in Expt. 3 (b), containing 0.8 g. starch was diluted to 100 ml. with distilled water. The solution was warmed to 60°C, with passage of nitrogen, and powdered thymol was added to saturate the solution. This mixture was stirred for 30 minutes at 55-60°C and finally allowed to cool to room temperature with constant stirring in an inert atmosphere.

After two days, a copious white thymol-polysaccharide precipitate was deposited. This was removed on a high-speed

centrifuge and dispersed with stirring, under nitrogen, in distilled water at 90°C.

The centrifugate was freed from thymol by extraction with ether, and amylopectin (0.4 g.; 50%) isolated from the aqueous solution by freeze-drying after dialysis. It had $[\alpha]_D$+197° (c. 0.7 in water).

The amylose-thymol precipitate was dissolved in water and the solution saturated with n-butanol (10%) at 60°C. The mixture was allowed to cool to room temperature with stirring, then stoppered and set aside overnight. The deposited amylose-butanol complex was removed at the centrifuge, dissolved in water, and subjected to two more recrystallisations with n-butanol in the same way. It (160 mg.; 20%) was finally isolated by freeze-drying the aqueous solution after dialysis. It had $[\alpha]_D$+158° (c. 0.7 in molar potassium hydroxide).

Expt. 5 **Fractionation of the Starch-Type Glucan from the Green Seaweed Ulva lactuca**

The sample of freeze-dried starch (1.2% of dry weight of weed, kindly given by Dr. J. K. Wold) had ash (1.0%), protein (2.5%) and was not completely soluble in water. It (0.45 g.) was almost completely dissolved in sodium hydroxide (0.5 molar) after stirring for 12-20 hours in an atmosphere of nitrogen.

A small insoluble residue was removed by filtration and the alkaline extract was dialysed to neutrality. The starch solution obtained in this way was quite clear, although it

showed some tendency to precipitate at low dilution. It (0.4 g., determined by cuprimetric titration[80]) had $[\alpha]_D$ +191° (c. 0.2, in water) and gave a blue colour with iodine.

The solution was concentrated to 100 ml. and subjected to the treatment with thymol and n-butanol as described previously for C. rupestris starch (Expt. 4), to give an amylose (146 mg.; 37%) and an amylopectin (200 mg.; 50%) fraction.

Expt. 6 **Isolation of a Starch-Type Polymer from Chaetomorpha capillaris**

A hot-water, freeze-dried extract (11.0 g.) of the green weed Chaetomorpha capillaris which had been treated with trichloroacetic acid, was found to give a purple stain with iodine.

By application of the iodine precipitation method as described in Expt. 3, a small amount (200 mg.) of starch-type glucan was isolated. This material had $[\alpha]_D$ +161° (c. 0.1 in molar sodium hydroxide), and gave a purple colour with iodine.

The absence of the intense blue colour characteristic of the amylose fraction of starch suggested that, in this case, no amylose was present. This was supported by the failure to obtain a precipitate with thymol. Since this polysaccharide extract had been obtained by treatment with boiling water under aerobic conditions and subsequently with trichloroacetic acid, it seems likely that the amylose component had undergone

extensive degradation. It is certainly probable that an amylose component was originally present, since a hot-water extract of the native weed gave an intense blue colour with iodine.

The remainder of this experimental section describes the methods used to characterise the components of these algal starches, in studies carried out concurrently with the analogous fractions of potato (Duke of Kent) starch, kindly provided by Mr. J. R. Stark.

The results of the preliminary investigations (Expt. 1-6) are summarised in table 2.

Glucan Source	$[\alpha]_D$	Iodine Colour	Amylose (%)
C. rupestris	+193°	blue	20
U. lactuca	+191°	blue	37
Ch. capillaris	+161°*	purple	-
Potato	+201°	blue	25

table 2

* measured in molar sodium hydroxide

Expt. 7 Examination of the Algal Amyloses and Amylopectins

Portions of each freeze-dried glucan (ca. 10 mg.) were hydrolysed with sulphuric acid (2N; 1 ml.) for 2.5 hours at 100°C. Aliquots of the hydrolysates were neutralised with sodium

Amyloses from:	$[\alpha]_D$ [1]	Iodine Colour	Purity (%) (cuprimetric)	Purity (%) (phenol-H_2SO_4)
Cladophora	+158°	blue	75	92
Ulva	+161°	blue	73	94
Potato	+157°	blue	78	94

table 3a

1 measured in molar potassium hydroxide

Amylopectins from:	$[\alpha]_D$	Iodine Colour	Purity (%) (cuprimetric)	Purity (%) (phenol-H_2SO_4)
Cladophora	+197°	purple	81	83
Ulva	+205°	purple	86	86
Chaetomorpha	+161° [2]	purple	-	79
Potato	+197°	purple	94	94

table 3b

2 measured in molar sodium hydroxide

hydroxide (0.1 N) and the respective glucose contents found by cuprimetric titration.[80] Standard solutions of each glucan were also prepared (alkali was required to dissolve the freeze-dried amyloses), and aliquots of these were removed and their glucose contents determined by means of the phenol-concentrated sulphuric acid reagent.[81] The values are given in tables 3a and 3b.

The remainder of the hydrolysates were de-ionised with Amberlite IR-120(H) and IR-45B(OH) ion-exchange resins, filtered and concentrated to syrups. Chromatographic examination of each (solvents 1,2 and 3) revealed the presence of a single sugar with the same mobility as authentic glucose run as control. The residual syrups were each incubated with glucose oxidase at 37°C for 24 hours, in phosphate-citric acid buffer.(0.05 molar; 0.1 ml.; pH 6). In each case, a product with the same chromatographic and ionophoretic mobilities of D-gluconic acid was obtained, together with some unattacked glucose.

Expt. 8 Viscosity Measurements on the Amylose and Amylopectin Fractions

The specific viscosity (η_{sp}) of a solution of a polymer depends upon the concentration of the solution. The viscosity number for any particular concentration is given by the ratio η_{sp}/c. The intrinsic or limiting viscosity is given by the relationship $[\eta] = \lim_{c \to 0} \eta_{sp}/c$, c = concentration(g./100 ml.) Values of $[\eta]$ are obtained from a graphical plot of the viscosity numbers (η_{sp}/c) against the concentrations. Extrapolation of

the straight lines back to the η_{sp}/c axis gives the required value.

In the present experiments, viscosity measurements were carried out in molar potassium hydroxide using an Ubbelohde viscometer. Specific viscosities (η_{sp}) were determined from the relationship $\frac{T-T_o}{T}$, where T is the flow time of the polymer solution, and T_o is the flow time of the solvent measured in seconds.

The experimental determinations of T and T_o were made as follows:-

A solution of the glucan (0.5-1%) was prepared by dissolution in molar potassium hydroxide in the presence of nitrogen gas. The exact concentration of polysaccharide was found by cuprimetric estimation in the usual way. All solutions were filtered by gravity through sintered glass (porosity 4) before the flow times were measured with the viscometer clamped vertically in a constant temperature bath at 25°C.

Solvent (10 ml.) was placed in the viscometer and the average flow time (T_o; 10 readings) established after a suitable time allowance for temperature equilibration of the apparatus. (at least 30 minutes). Polysaccharide solution was added successively in aliquots (4-5 ml.), and the new flow times (T) determined after each addition.

The experimental data obtained for Cladophora and Ulva amyloses and amylopectins are given in tables 4a-4d. The graphical estimations of $[\eta]$ obtained for these and potato glucans are listed in tables 5a and 5b.

Cladophora Amylose (T_0 126.75 secs.)

c. (g/ml.)	T (secs.)	$T-T_0$ (secs.)	η_{sp}/c
0.001607	142.87	16.12	79.09
0.002611	152.10	25.35	78.38
0.003298	160.37	33.62	80.37
0.003798	165.55	38.80	80.57

table 4a

Cladophora Amylopectin (T_0 224.15 secs.)

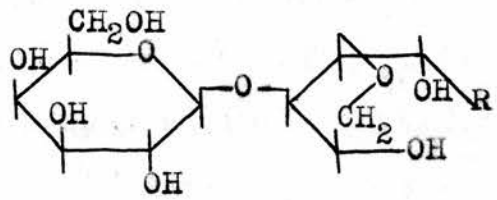
c. (g/ml.)	T (secs.)	$T-T_0$ (secs.)	η_{sp}/c
0.001370	237.32	13.17	42.88
0.002131	245.32	21.17	44.31
0.002616	250.62	26.47	45.13
0.002950	254.24	30.09	45.48

table 4b

Ulva Amylose (T_0 125.65 secs.)

c. (g/ml.)	T (secs.)	$T-T_0$ (secs.)	η_{sp}/c
0.001022	131.06	5.41	42.10
0.001662	134.63	8.98	43.00
0.002098	136.47	10.82	41.10
0.002416	138.51	12.86	42.70

table 4c



(a) R -CHO

(b) R $-CH(OCH_3)_2$

(c) R $-CH(SC_2H_5)_2$

fig. 8 - agarobiose

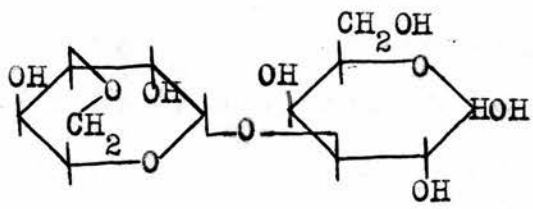


fig. 9 - neoagarobiose

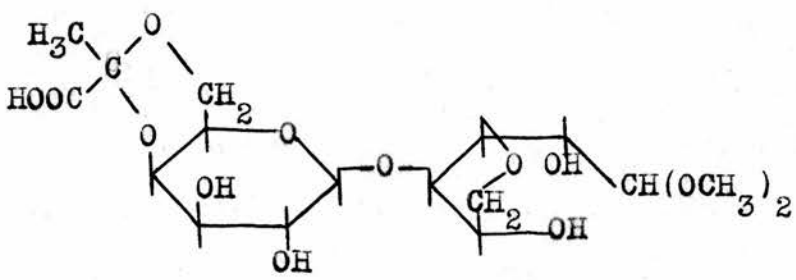
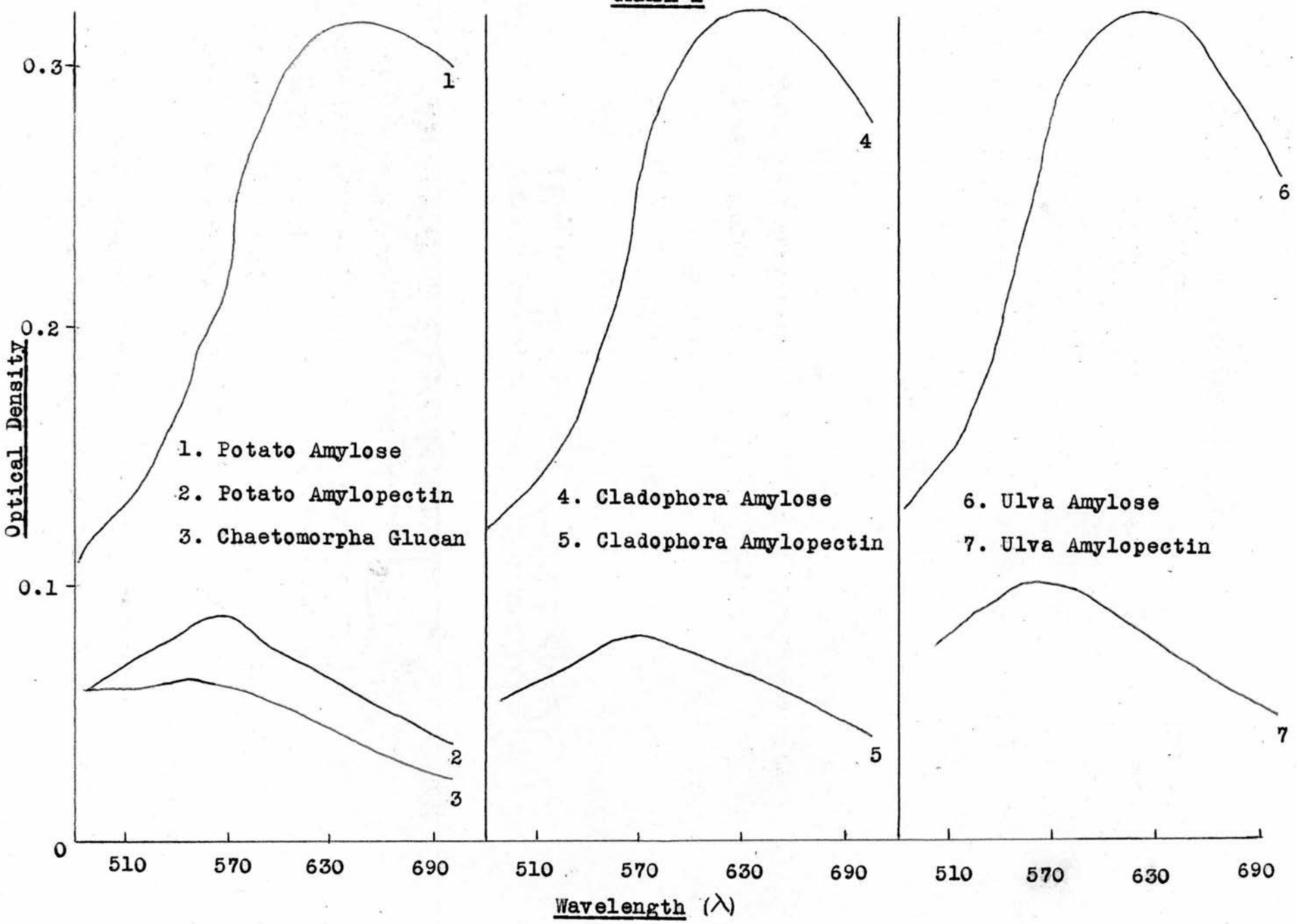


fig. 10

GRAPH 1


Optical Density
0.3
0.2
0.1
0
1
2
3
4
5
6
7
1. Potato Amylose
2. Potato Amylopectin
3. Chaetomorpha Glucan
4. Cladophora Amylose
5. Cladophora Amylopectin
6. Ulva Amylose
7. Ulva Amylopectin
510
570
630
690
Wavelength (λ)

Ulva Amylopectin (T_o 126.17 secs.)

c. (g/ml.)	T (secs.)	$T-T_o$ (secs.)	η_{sp}/c
0.000676	131.30	5.13	60.20
0.001050	134.13	7.96	60.10
0.001290	136.15	9.98	61.50
0.001450	137.89	11.44	62.30

table 4d

Amylose Molecules	
Glucan	Viscosity
Cladophora	78
Ulva	41
Potato	300

table 5a

Amylopectin Molecules	
Glucan	Viscosity
Cladophora	41
Ulva	58
Potato	161

table 5b

Expt. 9 Properties of the Glucan-Iodine Complexes [102]

Glucan solution containing a known quantity of polymer (5 mg.) as determined by the Somogyi [80] reagent, was transferred to a standard flask. (500 ml.). Distilled water (100 ml.), hydrochloric acid (6N; 0.25 ml.) and standard iodine solution (0.2% I_2 in 2% KI; 5.0 ml.) were then added followed by distilled water to the mark.

The spectrum of this solution was examined between 450 mμ. and 700 mμ. in a Unicam SP 500 spectrophotometer. (Graph 1).

The wavelength of maximal absorption (λ max) was taken as the mid-point of the peak obtained by plotting wavelength (λ) against optical density.

The blue value (BV) of the glucan-iodine complex is defined as the optical density (OD) at 680 mμ. in 4 cm. cells or 4 x OD in 1 cm. cells. The values of λ max and BV which were found for the various amylose and amylopectin fractions are given in tables 6a and 6b.

Amylose Molecules		
Glucan	λ max	BV
Cladophora	635	1.20
Ulva	630	1.14
Potato	640	1.22

table 6a

Amylopectin Molecules		
Glucan	λ max	BV
Cladophora	565	0.196
Ulva	560	0.220
Chaetomorpha	540	0.108
Potato	560	0.176

table 6b

Expt. 10 Enzymic Degradation of Glucan Fractions

(i) With α-amylase

The salivary α-amylase used in these experiments was purified according to the method of Fischer and Stein[103] and was kindly given by Dr. D. J. Manners.

Amylopectin samples (0.05-0.1%; estimated by Somogyi cuprimetric titration[80]), together with salivary α-amylase (0.1%) and sodium chloride (0.005%) were each incubated separately at 37°C in citric acid - phosphate buffer. (0.04 molar; pH 7.0; 20-25 ml.). A blank digest, in which water

replaced polysaccharide solution, was also prepared.

At intervals, aliquots of the digests were removed and analysed for their maltose contents by cuprimetric titration.

The apparent percentage conversion into maltose (P_M) values were then evaluated, and these are given in table 7.

Glucan	P_M(6 hrs.)	P_M(24 hrs.)
Cladophora	79	92
Ulva	85	91
Chaetomorpha	-	81
Potato	83	92

table 7

(ii) With β-amylase

(a) The enzyme used in these experiments was a commercial Wallerstein preparation known to possess Z-enzyme activity. (page 46).

It had an activity of 100 units/mg. as determined by the method of Hobson, Whelan and Peat[104], and was kindly given by Dr. D. J. Manners.

Amylose and amylopectin samples (0.05-0.1%; estimated by Somogyi cuprimetric titration[80]), together with Wallerstein β-amylase (0.1%) and serum albumin (0.01-0.05%) were each incubated separately at 37°C and pH 3.6 in acetate buffer. (0.04 molar; 20-25 ml.).

Similar digests were also prepared in solution buffered at pH 4.6. Blank digests, in which water replaced polysaccharide

solution, were also prepared.

Toluene (1 drop) was added to each digest to inhibit bacterial action.

After 48 hours, aliquots of each digest were removed and analysed for their maltose contents by cuprimetric titration. The apparent percentage conversion into maltose (P_M or β-limit) values are given in tables 8a and 8b.

Amylose Molecules

Glucan	β-limit (pH 3.6)	β+Z-limit (pH 4.6)
Cladophora	73	88
Ulva	71	90
Potato	80	100

table 8a

Amylopectin Molecules

Glucan	β-limit (pH 3.6)	β+Z-limit (pH 4.6)
Cladophora	52	57
Ulva	-	56
Chaetomorpha	-	62
Potato	53	56

table 8b

(b) The enzyme used in these experiments was a purified β-amylase preparation containing no maltase or Z-enzyme activity. (activity 3000 units/ml.)

Amylopectin samples were prepared in acetate buffer as described in Expt. 10a, and contained glutathione (0.5 molar) to stabilise the β-amylase.

Aliquots of each digest were removed and analysed for their maltose contents after 24 hours. These were used to evaluate true β-limits.

A small amount (5 mg.) of the Wallerstein β-amylase

preparation was then added to each digest which were then incubated for a further 24 hours at 37°C. Aliquots of these digests were withdrawn and their maltose contents re-determined and used to evaluate β+Z-limits.

A blank digest was prepared and examined in the same way.

Since glutathione interferes with cuprimetric estimation of reducing sugars, the sugar contents of these digests were found by ferricyanide-ceric sulphate titration.[105]

The P_M values obtained for the amylopectin molecules in this way, are given in table 9. This table also includes values obtained with a sample of Codium fragile amylopectin provided by Dr. J. Love.

	Amylopectin Molecules	
Glucan	β-limit (24 hrs.)	β+Z-limit (48 hrs.)
Cladophora	50	55
Ulva	51	55
Codium	51	53
Potato	53	57

table 9

Amylose Molecules

Time (hours)	Moles IO_4^- Reduced per C_6 Anhydro Unit		
	Cladophora	Ulva	Potato
20	0.488	0.424	0.684
44	0.699	0.748	0.891
68	0.900	0.937	0.991
98	0.972	1.01	1.01
170	0.991	1.09	1.01

table 10a

Amylopectin Molecules

Time (hours)	Moles IO_4^- Reduced per C_6 Anhydro Unit			
	Cladophora	Ulva	Chaetomorpha	Potato
20	0.671	0.630	0.862	0.844
44	0.890	0.700	0.976	0.964
68	1.02	1.03	1.09	1.03
98	1.05	1.05	1.11	1.04
170	1.07	1.16	1.18	1.08

table 10b

87

Expt. 11 Oxidation of the Amylose and Amylopectin Glucans with Sodium Metaperiodate

Polysaccharide samples (10 mg.) were oxidised with aqueous sodium metaperiodate (0.015 molar; 5 ml.) in the dark at 4°C.

At intervals, the reduction of periodate was measured according to the method of Aspinall and Ferrier. (page 41). It was found that the polysaccharides reduced approximately 1 mole of periodate per anhydroglucose unit.

The results are given in tables 10a and 10b.

When the oxidations were complete, ethylene glycol was added to stop the reactions. Potassium borohydride (10 mg.) was added to each and the solutions were left overnight at room temperature. The derived polyalcohols were isolated by freeze-drying after dialysis, and hydrolysed with sulphuric acid. Chromatographic examination of the neutralised hydrolysates indicated that no glucose residues had survived the periodate oxidations.

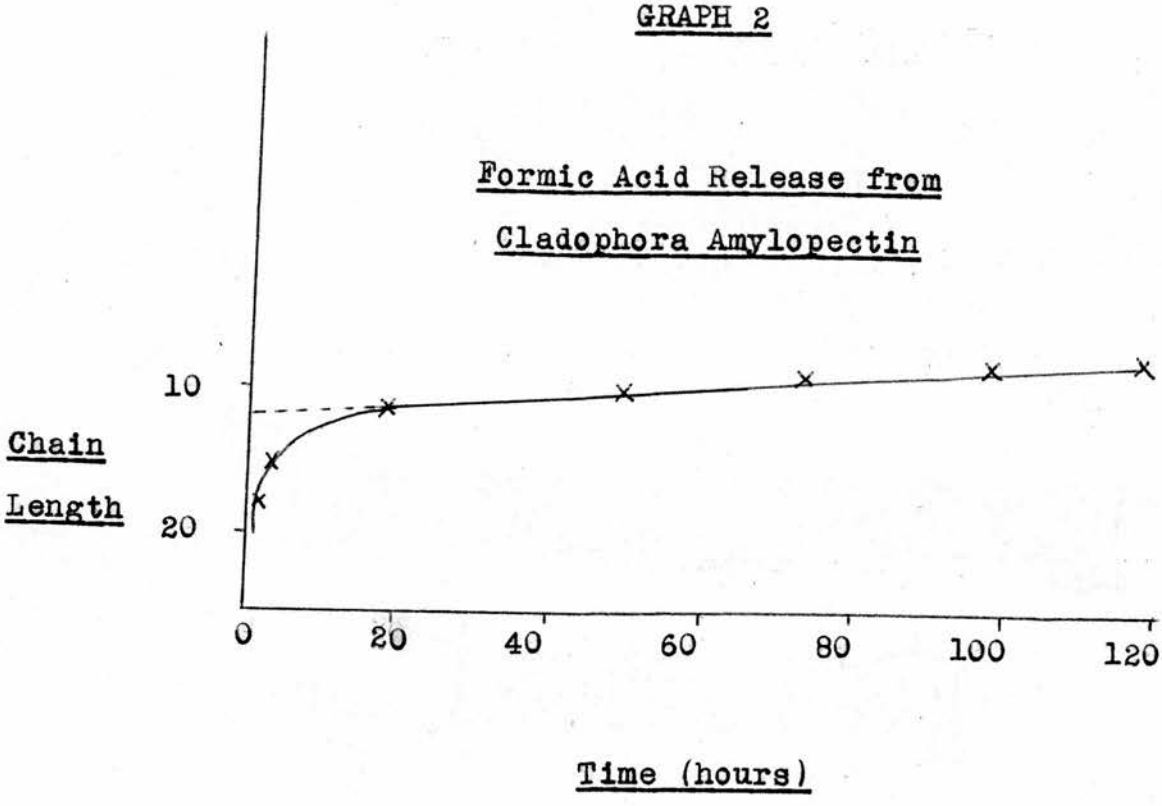
106

Expt. 12 Determination of the Average Chain Length of Cladophora Amylopectin by Oxidation with Sodium Metaperiodate

Polysaccharide (105 mg.) was dissolved in carbon dioxide-free water (20-30 ml.), and the pH of the solution adjusted to 5.8. Sodium metaperiodate solution was added to give a final concentration of 0.1 molar (50 ml.), and the mixture was stored in the dark at a constant temperature of 20°C.

At intervals, aliquots were removed and mixed with

GRAPH 2


Formic Acid Release from
Cladophora Amylopectin
Chain
Length
10
20
0
20
40
60
80
100
120
Time (hours)

ethylene glycol (0.5 ml.), and left in an inert atmosphere for 5 minutes.

The liberated formic acid was titrated potentiometrically with carbon dioxide-free sodium hydroxide (0.005025 N) to an end-point of pH 5.8.

The results are shown in table 11, values of total chain length being estimated from the following relationship:

Total Chain Length (TCL)

$$\frac{\text{wt. of amylopectin (g. in aliquot)} \times 1000}{\text{Titre} \times 0.005025 \times 162}$$

Time (hours)	NaOH (ml.)	TCL
1	0.71	18.2
3	0.84	15.4
18	1.11	11.6
25	1.14	11.3
49	1.23	10.5
73	1.38	9.4
97	1.46	8.8
117	1.55	8.3

table 11

The average chain length was found graphically (Graph 2) to be 12. The average total chain lengths found for potato and Codium amylopectin, in the same way, were 23 and 11 respectively.

Expt. 13 Estimation of Chain Length of the Amylopectin from Enteromorpha compressa

A small amount of the methyl glucosides from methylated E. compressa amylopectin (prepared by Dr. J. P. M^{c}Kinnell) was hydrolysed with sulphuric acid, and the derived neutralised syrup separated on Whatman No. 1 filter paper by elution in solvent 4.

The paper was dried after elution for 3 hours and eluted for a further 6 hours with the same solvent. The portions of paper containing the tri- and tetra-O-methyl sugars, together with comparable areas of blank paper, were eluted with water and methanol.

The eluates, after concentration to dryness, were dissolved in a known volume of water, and the sugar contents of measured aliquots were determined with the phenol-concentrated sulphuric acid reagent[81] by reference to standard graphs.

The molar ratio of tetra- to tri-O-methyl glucose was estimated to be 1 : 26; that is the average total chain length of the amylopectin was 26.

Expt. 14 Methylation of the Amylopectin Fraction from Codium fragile

Amylopectin (120 mg.) was dissolved in water (10 ml.) containing potassium borohydride (50 mg.), and the mixture left for 20 hours at room temperature.

To the amylopectin solution, sodium hydroxide (60%; 10 ml.) and dimethyl sulphate (6 ml.) were added dropwise over a period of 6 hours.

Throughout these additions, the mixture, saturated with nitrogen, was stirred vigorously and kept below 2°C. The mixture was then stirred at room temperature overnight.

The methylation was repeated twice under exactly the same conditions. The partially methylated material (100 mg.) was isolated after dialysis by freeze-drying, suspended in acetone/water (1/1) and subjected to three more methylations in the same way.

The partially methylated substance was isolated as before and extracted overnight with boiling chloroform in a Soxhlet thimble. The product (30 mg.) was dried overnight and stirred with freshly distilled methyl iodide, while dry silver oxide (2 g.) was added in portions (0.2 g./30 mins.). This mixture was refluxed overnight and another methylation effected in the same way. The product was filtered and the residue extracted with boiling chloroform and filtered.

The two filtrates were combined and concentrated to give a syrup which was soluble in methyl iodide. This material was subjected to two further methylations in the same manner.

The final product (15 mg.) was dried and refluxed with dry methanolic hydrogen chloride for 8 hours. After neutralisation with silver carbonate, filtration and concentration, the mixture of methyl glucosides was analysed by gas-liquid chromatography[85], whereupon peaks with the characteristic retention values of methyl 2,3,4,6-tetra-O-methylglucosides, methyl 2,3,6-tri-O-methylglucosides and methyl di-O-methylglucosides were detected.

Discussion

The green seaweed Cladophora rupestris has been the subject of two previous investigations in this laboratory. The first of these produced no evidence for the existence of a starch-type polysaccharide, although a tentative identification of a laminarin-type polymer was made.[73]

In a second examination, it was shown that a starch-type glucan could be isolated from the exhaustively extracted (hot water) weed residues, with alkali after a chlorite treatment, by formation of its iodine complex. The glucan obtained by this means revealed many anomalous properties as compared with potato starch, and it was considered that most of these were attributable to the drastic methods required for its isolation.[107]

The present investigations were therefore undertaken to isolate the starch-type glucan from Cladophora rupestris weed by a milder process, and to determine its constitution, and that of similar glucans isolated from Ulva lactuca and Chaetomorpha capillaris. Parallel experiments with potato starch were carried out, and the properties of this material were compared with those of the algal glucans.

It was found that treatment of the finely powdered decolourised Cladophora with boiling water, under anaerobic conditions, gave extracts which produced intense blue colours with iodine.

Application of the iodine precipitation process of Steiner and Guthrie[99] allowed separation and purification of the starch-

type polymer from the residual sulphated material. (Expt. 3).

The same method was also successfully used to obtain starch-type polysaccharides from freeze-dried, hot-water extracts of Ulva lactuca (by J.K. Wold) and Chaetomorpha capillaris, provided by Dr. E. E. Percival.

The materials from Cladophora and Ulva gave intense blue colours with iodine, whereas that from Chaetomorpha produced a reddish-purple colour. Each polymer had a high positive rotation (ca. $+200^{\circ}$ in water, $+160^{\circ}$ in alkali), comparable with potato starch and gave only glucose on acidic hydrolysis. The glucans from Cladophora and Ulva were contaminated with a small amount of ash and nitrogenous substances (probably protein), and it was likely that the Chaetomorpha glucan possessed similar impurities.

The colours of the iodine complexes of the glucans suggested that those of Cladophora and Ulva contained an amylose component, whereas that of Chaetomorpha did not. This was confirmed by fractionation experiments with thymol. (Expts. 4 and 5). It is well known that the amylose component of starch is able to form water-insoluble complex compounds with minor proportions of slightly soluble polar organic substances, such as thymol and butanol.[91,100,108] The failure to obtain a precipitate may indicate that the amylose is too small a molecule to form a complex, or that no amylose is present.

It is essential that the conditions of precipitation are chosen so that the first separation gives amylopectin of maximal purity, since further removal of amylose contaminants

from the first supernatant is not easy. Pure amylose samples are more readily obtained, since they can be recrystallised from solutions of n-butanol.[91]

In the present experiments, amylose fractions were obtained from Cladophora and Ulva, after three recrystallisations from n-butanol, to the extent of 20% and 37% respectively of the original weight of starch. (cf. potato, ca. 25%). No amylose complex could be formed from Chaetomorpha glucan.

It should be noted that the yields of amyloses cannot be strictly quantitative from these fractional precipitations, so that the values found probably represent minimum amylose contents. This method of fractionation is also liable to extensive losses of polysaccharide, particularly in the supernatant solutions from the fractional precipitations, where material whose structure is between that of linear amylose and amylopectin may occur.[91] Losses may also have taken place in the ensuing dialysis.

The amylose and amylopectin fractions gave only glucose on acidic hydrolysis. This was identified chromatographically and by its conversion to gluconic acid in the presence of the specific enzyme glucose oxidase. (Expt. 7).

Portions of each glucan were hydrolysed and the resultant glucose estimated by cuprimetric titration after neutralisation.[80] Some difficulty was experienced here, since the freeze-dried amylose samples apparently resisted complete hydrolysis under the standard conditions. (2.5 hrs. at 100°C with 2 N sulphuric acid). This would account for the low, though nevertheless

comparable, estimates of percentage purity obtained for the algal and potato amyloses. (table 3a). The hydrolyses of the respective amylopectin samples proceeded smoothly under the stated conditions, and the figures obtained show that the algal amylopectins were rather less pure than the corresponding potato amylopectin.(table 3b)

Determinations of the glucose contents of the amyloses and amylopectins were also made with the phenol-concentrated sulphuric acid reagent.[81] The values found for the amyloses were higher than those from cuprimetric estimation and were again comparable with each other. (table 3a). The amylopectin figures were the same as the cuprimetric figures. (table 3b).

In view of the scarcity of material and the difficulties attending the recovery of further samples of seaweed at the time of year, no further efforts were made to purify these glucan fractions.

The limiting viscosities ($[\eta]$) of starch components, especially amyloses, are considered to be a reflection, to some extent, of the molecular size of the polymers, and it has been suggested that for amyloses of DP up to 4000, the relationship $DP = 7.4 \times [\eta]$ may be applied.[105] (DP is the degree of polymerisation).

Measurement of the limiting viscosities of the algal and potato amyloses and amylopectins was carried out in molar potassium hydroxide under standard conditions. (Expt. 6). In each case, the limiting viscosity of the algal glucans was

much less than the corresponding values for potato fractions.

The following results were obtained:-

Amylose Molecules			Amylopectin Molecules	
Glucan	Viscosity	DP	Glucan	Viscosity
Cladophora	78	577	Cladophora	41
Ulva	41	302	Ulva	58
Potato	300	2220	Potato	161

If the above relationship can be applied, then clearly the algal amyloses must be smaller molecules than potato amylose. Furthermore, since the algal and potato starches were isolated under comparable conditions, it is not unreasonable to suppose that this difference is a real one, and not merely an effect of degradation during isolation. The implications of the limiting viscosities of amylopectins are more obscure, although it is believed that the shape of the molecule is important in influencing this. For example, it has been suggested that glycogen (limiting viscosity ca. 10) is a more compact, spherically shaped molecule than potato amylopectin, and that this is the most likely reason for the great difference in limiting viscosity between these two polymers.[109]

It is surprising that the magnitudes of the limiting viscosities of the Ulva starch components are reversed (i.e. amylose < amylopectin). However, it is doubtful whether any real significance can be attached to this.

One of the most characteristic properties of starch-type polysaccharides is their ability to interact with iodine to form coloured complexes. The great difference in degree to which iodine is bound by amylose and amylopectin has long been a major factor in their distinction. There is some evidence that the iodine staining power of amylose is related to the degree of polymerisation, provided that the amylose molecules do not exceed a certain critical size.[110,111] It has also been suggested that a relationship exists between the iodine absorption spectra in ammonium sulphate solution and the lengths of the interior chains of amylopectin and glycogen molecules.[112]

The amylose is thought to exist in a helical configuration in which the iodine molecules are arranged in an axial and end-wise fashion. In addition, it is believed that each coil of the helix is associated with one iodine molecule, and is made up of six glucose units. Experiments with synthetic amyloses have indicated that the minimum degree of polymerisation required to produce an iodine stain is eighteen glucose units; that is a sequence of three coils is needed.[110]

In the case of the amylopectin complex, Higginbotham has suggested that iodine is bound partly by the helical mechanism and partly by the absorption of iodine molecules or tri-iodide ions.[113]

Two properties of the iodine complex are normally considered, namely the wavelength at which absorption is a maximum (λ max), and the blue value. (BV). Each of these properties is useful

to distinguish between amyloses, amylopectins and glycogens. Amyloses show a maximum absorption in the region 640-680 mμ., and have blue values from 1.2-1.5. Amylopectins absorb maximally in range 515-560 mμ., and have blue values from 0.1-0.15. Glycogens show broad maximum absorption bands within the range 420-500 mμ., while their blue values are less than 0.1.[114,115]

The following values of λmax and blue value were found for the algal and potato amylose and amylopectin fractions under the standard staining conditions. (Expt. 9).

Amylose Molecules			Amylopectin Molecules		
Glucan	λmax	BV	Glucan	λmax	BV
Cladophora	635	1.20	Cladophora	565	0.196
Ulva	630	1.14	Ulva	560	0.220
			Chaetomorpha	540	0.108
Potato	640	1.22	Potato	560	0.176

These results provide strong evidence for the similarity of the algal and potato starch components. The seaweed amyloses, particularly that of Ulva have slightly lower λmax and blue values than potato amylose and this may be correlated to some extent with their lower DP as found from the limiting viscosity numbers. However, the correlation should be regarded with caution in this case, since Bailey and Whelan have pointed out that the iodine staining qualities of amyloses of DP ca. 300 differ little from those of DP several thousand.[110] It is also possible that contamination of the amyloses with a small amount of amylopectin would produce similar changes in

iodine binding properties. However, it is felt that this is a less likely possibility in the present instance, since the amyloses were subjected to three recrystallisations from n-butanol before examination.

To judge from the rather high blue values of the amylopectins, apart from Chaetomorpha, it does seem probable that these samples do contain a small amylose impurity. This is not surprising if the algal amyloses are relatively small molecules, since they might be incompletely precipitated by thymol, and so contaminate the amylopectin fraction. Similarly, the potato amylopectin may be contaminated with smaller amylose chains.

The specific manner in which α- and β-amylases degrade α-1,4- linked glucans to maltose has already been described in an introduction to this section. (page 45).

Digestion* of the amylopectin molecules under the stated conditions (Expt. 10 (i)) gave the following P_M (percentage conversion to maltose) values after 24 hours:-

Glucan	P_M	
Cladophora	92	
Ulva	91	*with α-amylase
Chaetomorpha	81	
Potato	92	

The lower figure obtained for Chaetomorpha amylopectin suggests a slight structural deviation in this case, possibly due to degradation.

More precise information regarding the structures of the amylose and amylopectin molecules were obtained from studies with the specific enzyme β-amylase. Two preparations of β-amylase were used. Neither of these possessed maltase activity, but the commercial Wallerstein sample did have a Z-enzyme impurity.

P_M values with Wallerstein β-amylase for amylose samples were found after incubation for 48 hours at pH 3.6 and pH 4.6. (Expt. 10 (ii)a).

At pH 3.6, where Z-enzyme is inhibited, degradation to the extent of 71% (Ulva), 73% (Cladophora) and 80% (potato) occurred. (table 8a; page 62). These low conversions to maltose are in accord with the observations of other workers that amyloses are resistant to complete β-amylolysis.[96] On the other hand, digestion at pH 4.6, where Z-enzyme is effective, resulted in values of 90% (Ulva), 88% (Cladophora) and 100% (potato). The algal β+Z-limits are still lower than expected for nearly linear molecules. Although contamination with amylopectin might explain this, again it is felt that this is unlikely because of the purification procedure employed. The low figures are more probably due to incomplete degradation of the seaweed amyloses because of their great tendency to retrograde from the digest media, even when solutions were prepared from the amylose-butanol complex. This, of course, might also partly account for the low values also obtained at pH 3.6. This provides further indirect evidence that the algal amyloses are small molecules, in accordance with the

observations of Whistler and Johnson that short potato amylose chains are unstable in aqueous solution.[116]

It is a general observation that degradation of amylopectin with β-amylase in the presence of Z-enzyme causes only a small additional increase in the production of maltose, as compared with the production of maltose with β-amylase alone.

This was verified for Cladophora and potato amylopectin by digestion with Wallerstein β-amylase at the two pH values, when the following results were obtained. (Expt. 10 (ii)a).

Glucan	β-limit (pH 3.6)	β+Z-limit (pH 4.6)
Cladophora	52	57
Potato	53	56

Comparable digestions of Ulva and Chaetomorpha amylopectins at the higher pH value gave P_M figures of 56% and 62% respectively. (table 6b; page 62). Once again, Chaetomorpha is a little different from the other algal and potato amylopectins, although the value of 62% is still typical of an amylopectin molecule.

The similarity between potato and the algal amylopectins (including Codium fragile), was confirmed in studies with a purified β-amylase sample containing no Z-enzyme activity. (Expt. 10 (ii)b). Digestion of the amylopectins with this enzyme gave the following apparent yields of maltose after 24 hours.

Glucan	Cladophora	Ulva	Codium	Potato
β-limit	50	51	51	53

These values are considered to be true β-limits and are comparable with the β-limits obtained with Wallerstein enzyme

at pH 3.6.

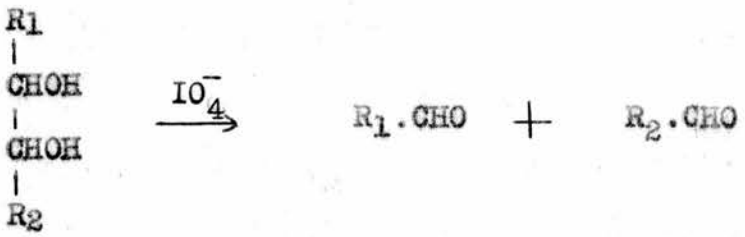
A small amount of Wallerstein enzyme preparation was then added to each digest and these were incubated for a further 24 hours. After this time, the measured maltose yields were:-

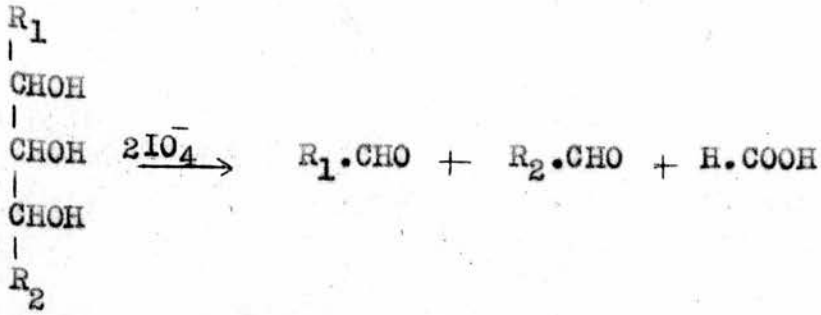
Glucan	Cladophora	Ulva	Codium	Potato
β+Z-limit	55	55	53	57

These figures are in good agreement with the values obtained with Wallerstein enzyme alone at pH 4.6. (table 8b; page 62).

The results of these enzymic studies support very strongly the classification of these algal glucan fractions as amyloses and amylopectins.

The effect of periodate on carbohydrate structures has been studied intensively in recent years, and it is known that periodate will cleave the 1,2-diol and 1,2,3-triol systems which commonly occur in sugars.[117] The first products are aldehydo compounds and often formic acid, which can arise by oxidation of substances containing triol groupings. The reactions may be depicted in the following general form:-





By careful choice of conditions, for example, absence of light, low temperatures and acidic pH values, cyclic systems can be oxidised selectively, and estimations of the amount of periodate reduced and the products formed may give a quantitative measure of the number of diol or triol systems in the molecule.

Reduction of the sugar-aldehyde products with borohydride to the corresponding more stable alcoholic substances, followed by acidic hydrolysis, if required, may give valuable information concerning the structure of the parent compound.

It is also known that, under certain conditions, over-oxidation, particularly with polysaccharides, may take place. This results in the reduction of greater quantities of periodate, and is often accompanied by the formation of distinctive products such as carbon dioxide and formaldehyde, as well as increased quantities of formic acid. In general, overoxidation takes place at alkaline pH values, (e.g. pH 8), at elevated temperatures, in the presence of large excesses of oxidant or in sunlight.[118,119] Careful use of overoxidation conditions can often provide invaluable information to add to that obtained by selective oxidation.[118]

In the present work (Expt. 11), overoxidation reactions were minimised by carrying out the oxidations in the dark at 2°C.

Nearly one mole of periodate was reduced for each anhydroglucose unit in the polysaccharides, and in no case was glucose found to have survived the oxidation. This is in accord with the accepted structures of amylose and amylopectin as consisting of 1,4- linked glucose units with branching points through C_6 of some of these. The possibility that the algal glucans contained significant amounts of 1,3- linkages (cf. floridean starch, page 7) was excluded, since these units would not have been cleaved in the oxidation.

Periodate oxidation of branched polymers such as glycogen and amylopectin will result in the liberation of formic acid from each non-reducing, terminal glucose residue. Formic acid will also be produced from the sole reducing group in the molecule, but this will constitute a neglible fraction of the total amount. The number of anhydroglucose units which correspond to one mole of formic acid released will therefore be an estimate of the average total chain length of the molecule.

The average chain length of amylopectins (ca. 20-25) and glycogens (ca. 9-14) is a property often used to classify these two types of polysaccharide.[120]

Normally, oxidations are carried out under conditions where overoxidation is kept to a minimum, so that a true estimate of formic acid from the non-reducing end residues is obtained.[120] However, experiments have also been described in which overoxidation is allowed to take place. Average chain length values may then be plotted against the time of oxidation and the true average chain length found by extrapolating the steady

portion of the graph back to zero time.[106]

By this method, Cladophora amylopectin had an average total chain length of 12 glucose units. (Graph 2, facing page 65). Similarly, potato and Codium amylopectins had chain lengths of 23 and 11 glucose units respectively. These values are in good agreement with 24 and 9, found in experiments where overoxidation did not take place.[28]

It is rather difficult to believe that the algal amylopectins possess such short glycogen-like average chain lengths in view of their similarity to potato amylopectin in other respects. One possible explanation may lie with the nature of the impurity in the algal amylopectins. If this is protein, then it is possible that some sort of weak buffering action may be induced. In this way, larger volumes of sodium hydroxide would be needed to reach an end point, and this would result in correspondingly low average chain length values. It may be pertinent, in this respect, that first reports of chain length for impure samples of floridean starch (page 7) were also of this low order, although this polymer possessed other properties more typical of amylopectin.

It should be mentioned, however, that other examples of the amylopectin-glycogen class of polymers possessing relatively short average chain lengths (9-15), and yet large β-limits, (50-60%), more typical of amylopectins, have been found, for example, in baker's yeast,[121] in the culture medium of the bacterium, Neisseria perflava,[122] in extracts of the protozoa, Trichomonas foetus,[123] and Trichomonas gallinae,[123] and in the unicellular salt

water alga, Dunaliella bioculata.[124] On the other hand, amylopectin molecules from the protozoal species, Chilomonas paramecium,[125] Polytomella coeca,[126] Holotrich ciliates,[127] Cycloposthium,[127] Polytoma uvella,[128] and Entodinium caudatum,[129] as well as the fresh water green alga, Nitella translucens,[130] have been found to resemble potato amylopectin in having higher chain length values. In addition, an average chain length of 26 was found by end-group analysis of the methylated amylopectin from the green seaweed, Enteromorpha compressa (Expt. 13), while values of about 21 were found both by formic acid release and methylation methods for the amylopectin-like glucan from another green seaweed, Caulerpa filiformis.[131]

It is evident that further research on this aspect of algal amylopectins will be required.

The final experiment in this section describes a methylation of a small amount of the amylopectin from Codium fragile. The methods involved were rather tedious and resulted in large losses of material. Gas-liquid chromatographic examination of the mixture of methanolised, methylated sugars, showed the presence of peaks corresponding to the methyl glycosides of 2,3,4,6-tetra-O-methyl glucose and 2,3,6-tri-O-methyl glucose.[85] The examination also showed that two methyl di-O-methyl glucosides were present in the mixture. Since one of these probably arose from undermethylation, and since the final recovery of methylated product was small and could have arisen from preferential methylation of low molecular weight material,

Properties of Amylose-Type Molecules

	Cladophora rupestris	Ulva lactuca	Enteromorpha compressa [1]	Codium fragile [2]	Potato
% in Starch	20	37	22	16	25
[3]	78	41	44	36	300
DP	577	302	325	266	2220
Blue value	1.20	1.14	0.66	0.96	1.22
λ max	635	630	610	610	640
$[\alpha]_D$	$+158^{\circ}$ *	$+161^{\circ}$ *	$+177^{\circ}$	$+197^{\circ}$	$+157^{\circ}$ *
β-limit	73	71	-	84	80
β+Z-limit	88	90	76	96	100
Moles IO_4^- reduced per anhydro unit	0.97	1.09	1.08	1.10	1.01

table 12a

* measured in molar potassium hydroxide

1 McKinnell, reference 3.

2 Love, reference 3.

Properties of Amylopectin Molecules

	Cladophora rupestris	Ulva lactuca	Chaetomorpha capillaris	Codium fragile [1]	Enteromorpha compressa [2]	Caulerpa filiformis [3]	Potato
[3]	41	58	-	24	35	15	161
Blue value	0.196	0.220	0.108	0.220	0.140	-	0.176
λ max	565	560	540	560	550	540	560
	$+197^{o}$	$+205^{o}$	$+161^{o}$ *	$+192^{o}$	$+190^{o}$	$+157^{o}$	$+197^{o}$
α-limit	92	91	81	84	90	90	92
β-limit	50	51	-	51	-	-	53
β+Z-limit	55	55	62	53	58	57	57
Moles IO_4^- reduced per anhydro unit	1.05	1.05	1.11	1.20	0.90	0.95	1.04

table 12b

* measured in molar sodium hydroxide

1 Love, reference 3.

2 McKinnell, reference 3.

3 Mackie, reference 131.

an estimation of the ratio of the tetra-O-methyl and tri-O-methyl glucoses was not made. (cf. Expt. 13).

Conclusion

The isolation of starch-type polysaccharides from Cladophora rupestris, Ulva lactuca and Chaetomorpha capillaris is in keeping with the accumulating evidence[3] and the X-ray studies of Meeuse and Kreger,[132,133] that starch-type polymers constitute the reserve carbohydrate material of green algae. In this respect, they appear to be similar to red algae (floridean starch),[15] blue-green algae, for example, Oscillatoria species,[134] and to many of the protozoa, as well as to land plants.

The properties of the amylose and amylopectin molecules from the present study are shown in tables 12a and 12b, together with those of other green seaweed starches for comparison.

It is interesting that apart from Ulva, which is higher (37%), the proportion of amylose (16-22%) in the other genera is comparable with that of the majority of land plant starches (20-30%), although certain varieties of pea[135] and maize[136] starches have a much higher amylose content.

It has been demonstrated that treatment of amylose components with boiling water in the presence of oxygen causes degradation of these molecules,[101] and it is probable that such effects account for the failure to isolate amyloses from the

genera Chaetomorpha capillaris and Caulerpa filiformis.[131]

That the native Chaetomorpha capillaris contained an amylose component was deduced from the deep blue colour given with iodine by solutions obtained after extraction of the weed for a short period with boiling water. (Expt. 6). Successive samples taken during a prolonged extraction with boiling water showed a progressive change in the intensity of the stain given with iodine, to a faint purple-pink colour more typical of an amylopectin. A similar effect was observed with the extracts of Cladophora rupestris and the related genus Chaetomorpha linum.

The seaweed amyloses are comparable in many ways with potato amylose, and undoubtedly possess an essentially linear structure of α-1,4- linked D-glucose units. It would appear, however, that they are much smaller molecules than potato amylose, although their degrees of polymerisation (300-600) are comparable with those of amyloses isolated from other sources, for example, maize,[137] malted barley,[138] parsnip,[130] Nitella translucens[130] and the protozoon, Chilomonas paramecium.[125] It should always be kept in mind however, that inadvertent degradation during isolation is a possible contributing factor towards the low DP of these molecules.

The algal amylopectins show even more striking similarity with potato amylopectin. Their rotations, properties of the iodine complexes and α- and β-limits are the same as potato amylopectin, and like the latter they reduce approximately one mole of periodate for every anhydroglucose unit.

The algal amylopectins do show some apparent anomalies in their viscosity numbers and average chain lengths found from the release of formic acid on periodate oxidation. As with the amyloses however, it is felt that the viscosity numbers are probably not so much a reflection of a difference of fundamental structure as a difference in molecular size, while it has already been suggested that further study will be required before major significance can be attached to the chain length values.

The author would like to point out that the work described in this section has been published in the Journal of the Chemical Society, and a reprint of this can be found inside the back cover of this thesis.

Part Two

The Water-Soluble Sulphated Polysaccharides of the Green Seaweeds

Cladophora rupestris, *Chaetomorpha linum* and *Chaetomorpha capillaris*

Experimental

The specimen of Chaetomorpha linum was harvested in Holland in November, 1961.

The samples of sulphated polysaccharide from Cladophora rupestris and Chaetomorpha capillaris were the materials isolated after removal of the starch-type polysaccharides. (Part One, pages 48-54).

Expt. 15 Extraction of the Water-Soluble Polysaccharides of Chaetomorpha linum

The seaweed (50 g.; dry weight) was frozen with liquid nitrogen, crushed to a powder and treated successively with aqueous n-butanol (half-saturated with water) and cold acetone, until no more colouring matter was removed.

The residual weed was isolated after centrifugation, and dried to a grey powder. The green organic solutions were concentrated to a smaller volume and extracted with ethyl acetate and water (1/1; 500 ml.). The aqueous layers were concentrated to dryness, hydrolysed and examined chromatographically whereupon traces of galactose, glucose and arabinose were observed. No polysaccharide material could be precipitated from this solution with excess ethanol.

The grey powder was extracted exhaustively, in turn, with cold and hot water (at 95°C), the latter procedure being carried overnight in an atmosphere of nitrogen. After a few hours, the hot-water extracts gave a blue colour with iodine, evidence that a starch-type polymer was present. Unfortunately,

during the extraction, the supply of nitrogen gas failed, and it was found that the final extract gave only a pale pink colour with iodine. (cf. Cladophora ; Expt. 2, page 50).

The hot-water extract was therefore clarified by centrifugation and digested with salivary ∝-amylase (page 45) to remove degraded starch-type polymer. On concentration to a small volume, this extract formed a stiff gel (cf. Cladophora; Expt 2, page 49), and this could only be dispersed satisfactorily by addition of trichloroacetic acid to a concentration of 4% at pH 4.

After 48 hours at 0°C, a dark brown slimy precipitate was removed in a Sharples high-speed centrifuge. The pH of the supernatant was then adjusted to 10 with sodium hydroxide, and set aside at 0°C for a further 48 hours. A similar precipitate was then removed in the same way as before. The cold-water and trichloroacetic acid treated hot-water extracts were dialysed to neutrality, and the solutions were examined further.

Expt. 16 Examination of the Cold-Water Extract of Chaetomorpha linum

The cold-water solution was divided into two portions (1) and (2). Portion (1) was treated with trichloroacetic acid as described above, and dialysed to neutrality after centrifugation. The neutral solution was subdivided further into two portions (1a) and (1b). Portion (1a) was freeze-dried directly to a light brown flaky solid (400 mg.)

Portion (1b) was treated with decolourising charcoal until a colourless solution was obtained, and the clear solution, after filtering, was freeze-dried to an off-white solid. (300 mg.)

Portion (2) of the original extract was freeze-dried directly without prior trichloroacetic acid treatment. It was a dark brown solid. (750 mg.).

These fractions had the following protein contents:-

Fraction	Protein (%)
1a	8.1
1b	4.9
2	10.6

table 13

They each gave the same proportions of monosaccharides on acidic hydrolysis (visual chromatographic examination) as follows:-

Sugar	Galactose	Arabinose	Glucose	Xylose	Rhamnose
Proportion	strong	strong	weak	medium	weak

In view of the small amount of the cold water soluble material (2.9% of dry weight) and its similarity to the hot-water extract (Expt. 18), no further work was carried out on this material.

The remainder of this experimental section contains a study of the starch-free sulphated polysaccharide obtained by treatment of the seaweeds with hot water. For convenience, these will hereafter be differentiated in the following manner:-

Cladophora rupestris	Polysaccharide (A_1)
Chaetomorpha capillaris	Polysaccharide (A_2)
Chaetomorpha linum	Polysaccharide (A_3)

Furthermore, after the preliminary examinations (Expts. 16-18), the experiments are presented under three broad headings as follows:-

1. Fractionation and Purification Experiments (pages 93-109).
2. Desulphation Studies (pages 109-119).
3. Partial Fragmentation Studies (pages 119-136).

The majority of the investigations deal specifically with polysaccharide (A_1) from Cladophora rupestris, since this was obtainable in greatest quantity. However, where it has been considered appropriate, analogous studies of the Chaetomorpha polysaccharides (A_2) and (A_3) have also been made.

It should perhaps be emphasised that the sample of polysaccharide (A_1) which had been treated with trichloroacetic acid (Expt. 2, page 49) was reserved solely for degradation experiments. All analytical and fractionation experiments were carried out on material which had not been pre-treated with trichloroacetic acid before isolation. (Expt. 3, page 50).

Expt. 17 Examination of the Starch-Free Polysaccharides (A_1), (A_2) and (A_3)

The polysaccharides had the following properties in table 14:-

Polysaccharide	Sugar (%)	$[\alpha]_D$	Sulphate[79]	Ash	Protein[78]
A_1	43.3	$+53^0$	12.3%	8.1%	25.6%
A_2	43.5	$+66^0$	15.2%	9.2%	19.4%
A_3	43.5	$+75^0$	15.5%	9.3%	18.8%

table 14

Percentage carbohydrate contents were obtained as follows:- Aliquots of standard polysaccharide solutions were treated with the phenol-concentrated sulphuric acid reagent.[81] The optical densities of the solutions were compared with aliquots of a standard solution treated in the same way. The standard solution contained galactose (38%), arabinose (42%), xylose (12%), rhamnose (5%) and glucose (3%).

The figures obtained cannot be regarded as absolute values, but are useful for comparison of different polysaccharide fractions.

Other Qualitative Tests

(a) 3,6-Anhydrogalactose[139] Polysaccharide solution containing about 10 mg. of polysaccharide was heated for ten minutes with resorcinol-hydrochloric acid reagent. (10 ml.) This was prepared immediately before use by mixing hydrochloric acid (s.g. 1.18; 200 ml.), resorcinol (0.13 g. in 200 ml. ethanol) and water. (40 ml.).

No crimson colours, such as was produced with authentic methyl 3,6-anhydrogalactoside, were obtained with polysaccharide (A_1), (A_2) or (A_3).

(b) Carbazole Test for Uronic Acid (by Dr. J. K. Wold)

Samples of polysaccharides (A_1) and (A_2) (10-20 mg.) were dissolved in water (50 ml.). Aliquots (2 ml.) were used for uronic anhydride estimation according to the method of McComb and McCready.[140] Values of 4% and 2.7% respectively were obtained. A sample of commercial starch, examined concurrently, gave a value of 9.5%, while a synthetic mixture of glucose, xylose and rhamnose gave a figure of 3.8%.

It was concluded that neither Cladophora nor Chaetomorpha hot-water extracts contained significant quantities of uronic acid. This was supported by the failure to detect uronic acids on chromatographic examination of their acid hydrolysates.

(c) Amino Sugar Residues[141] Polysaccharides (A_1), (A_2) and (A_3) (0.1 g.) were hydrolysed with sulphuric acid (2N; 10 ml.) at 100°C for 2.5 hours. The neutral hydrolysates were obtained in the usual way and made up to 5 ml. with distilled water.

Aliquots (1 ml.) of these solutions were analysed for amino sugar content, according to the procedure of Cessi and Piliego,[141] in which amino acids do not interfere. No red colours, such as were obtained with authentic glucosamine under the same conditions, were observed.

Paper chromatographic examination of the hydrolysates showed the presence of several amino acids (ninhydrin reagent),

including materials with the mobilities of glycine and leucine.

Expt. 18 Estimation of the Molar Proportions of Sugars in Polysaccharides (A_1), (A_2) and (A_3) [142]

Polysaccharides (50 mg.) were hydrolysed for 3 hours at 100°C with sulphuric acid, and neutral syrups obtained in the usual way. Aliquots of each syrup were applied to paper chromatograms.

Accurately measured volumes (10-50 µl.) of standard sugar solutions were also applied to paper chromatograms and eluted at the same time as those carrying the polysaccharide hydrolysates. (solvent 1; 18 hours).

The chromatograms were thoroughly dried in air before being dipped in a fresh solution of aniline phthalate, prepared by dissolving o-phthalic acid (1.66 g.) and redistilled aniline (0.91 ml.) in n-butanol (48 ml.), ethyl ether (48 ml.) and water. (4 ml.).

The dipped papers were dried in a current of air (3-5 hours), and then developed by heating at 105-110°C for 5 minutes.

The coloured spots were cut out, transferred to test-tubes, and eluted with 0.7N hydrochloric acid in 80% ethanol (4 ml.) for 1 hour. The optical densities of the solutions were read in a Unicam SP 600 visible spectrophotometer at 390 mµ (hexoses) and 360 mµ (pentoses), against a blank prepared by eluting a portion of the chromatogram which was free from sugar.

The weights of galactose, arabinose and xylose were found from a comparison of the optical densities of known weights of each sugar. The hydrolysates also contained small amounts

of glucose and rhamnose, but these were neglible in comparison with the other sugars, and were not estimated.

The proportions of sugars found are shown in table 15.

	Molar Proportions of Sugars		
Polysaccharide	Galactose	Arabinose	Xylose
A_1	3.1	3.2	1.0
A_2	1.4	3.7	1.0
A_3	1.8	3.7	1.0

table 15

1. Fractionation and Purification Experiments

Expt. 19 Chromatography of Polysaccharide (A_1) on DEAE (diethylaminoethyl)-Cellulose Paper

The following experiments were conveniently carried out in 100 ml. beakers containing the electrolyte (2-3 ml.), with copper wires as hanging rods. Solvent evaporation effects were eliminated by enclosure within glass covers.

Strips of Whatman DEAE-cellulose paper (10 x 2.5 cm.) were equilibrated by ascending capillary action of the salt solution used for subsequent elution. (30 minutes).

After the equilibration period, papers were removed and blotted lightly to remove excess solvent. Polysaccharide solution (0.5-1%) was applied as a spot, and the papers were immediately replaced in the eluting system for 15-20 minutes, then removed and dried in air. The chromatograms were washed successively with alcohol and ether to fix the

polysaccharides on the paper.

The sulphated polymer was located by dipping in toluidine blue (page 43; reagent 7) for 5 minutes. No satisfactory method of locating neutral polymers was found. Attempts were made to transfer polysaccharide areas to glass fibre strips, followed by development with anisaldehyde in concentrated sulphuric acid, but it was found that the chromatography paper itself was transferred just as readily. The regions of sulphated material generally showed up as dark blue spots or bands.

It was observed that polysaccharide displacements were readily effected with sodium chloride and sodium hydroxide solutions, provided the concentration of the former was at least 0.5 molar. Concentrations of sodium hydroxide less than 0.3 molar were sufficient to bring about definite migrations of sulphated polysaccharide. Incorporation of hydrochloric acid (pH 2) into the sodium chloride solutions gave similar results to sodium chloride alone.

However, no definite distinctions between bands of sulphated polysaccharide could be made because of the low resolution and the fact that a certain amount of the polysaccharide was apparently irreversibly held at the starting line.

Expt. 20 **Preliminary Experiments with Columns of Anion-Exchange Cellulose and Sephadex**

The DEAE-cellulose was Whatman DE-50 powder and the DEAE-sephadex was the Pharmacia A_{50} grade.

Prior to being packed into columns, these materials were

each subjected to the following preliminary treatment.

For example, DEAE-cellulose powder (300 g.) was stirred sequentially with hydrochloric acid (0.5 molar; 500 ml.), distilled water (until the supernatant was neutral), sodium hydroxide (0.5 molar; 500 ml.) and distilled water again.

Each treatment was de-aerated (water pump) and stirred for 30 minutes. The supernatant liquid was then removed by decantation when the bulk of the solid material had settled. Four such washing cycles were completed.

The DEAE-cellulose so prepared was converted into the chloride form by stirring with sodium chloride (0.5 molar; 2 x 500 ml.), followed by washing on a filter-pad with distilled water until no chloride ions were detected in the filtrate. The DEAE-cellulose obtained in this way was packed into columns as a slurry in the liquid to be used initially for elution.

Conversions into other anionic forms were effected by washing with a solution (0.5 molar) of the appropriate salt in the same way.

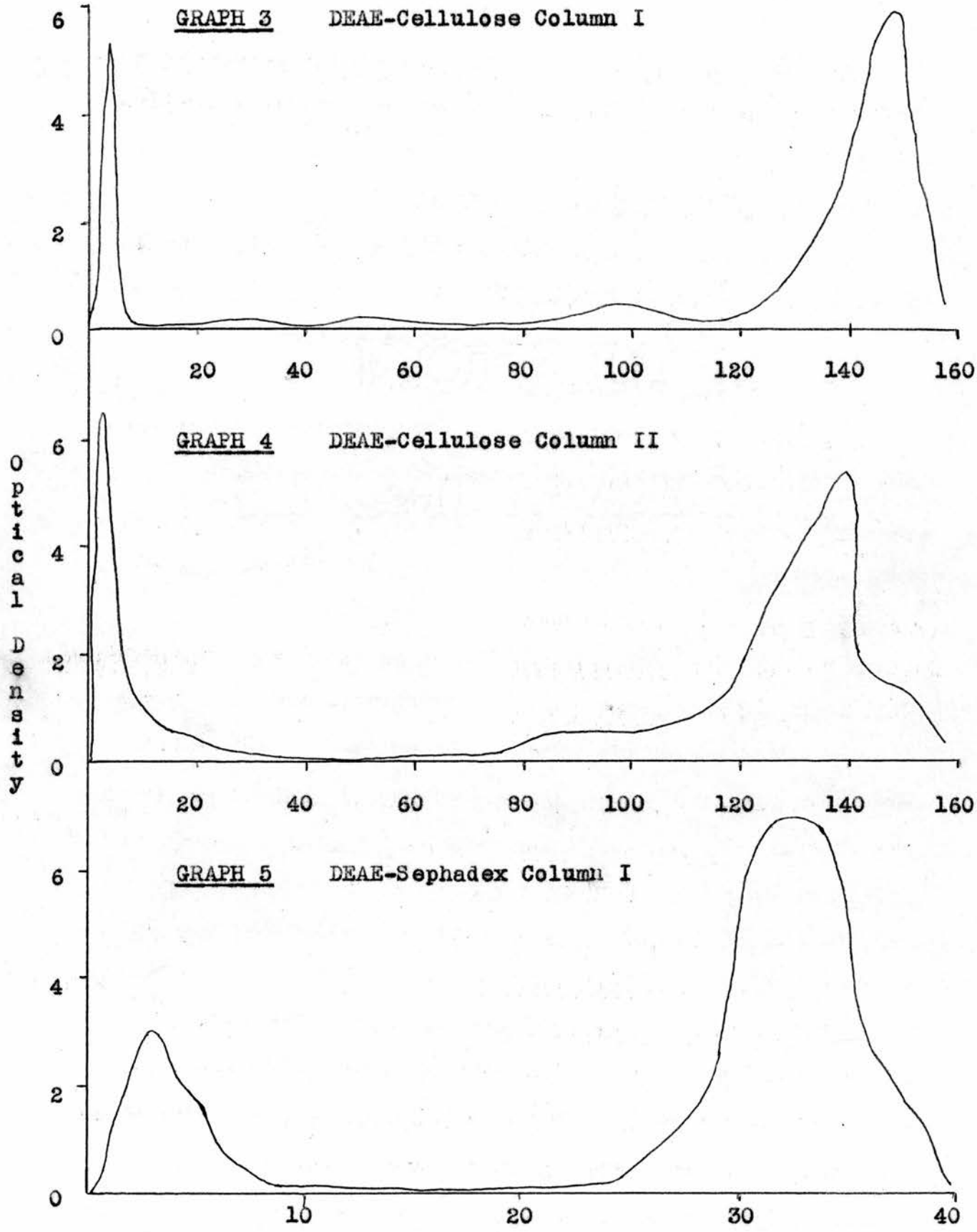
The abbreviations DC- and DS- have been chosen to distinguish DEAE-cellulose and DEAE-sephadex columns respectively. Similar columns are differentiated by the attachment of Roman numeral suffices; for example, DC-I and DC-II.

Initially, the following columns were prepared:-

(i) Column DC-I (6.0 x 3.0 cm.) was in the chloride form. Elution was carried out in two stages:-

1. 0.001 molar NaCl (300 ml.) $\longrightarrow$ 0.5 molar NaCl (300 ml.)

2. 0.5 molar NaCl + 0.01 molar HCl (250 ml.) $\longrightarrow$ 3.0 molar NaCl + 0.01 molar HCl (300 ml.)


GRAPH 3 DEAE-Cellulose Column I
GRAPH 4 DEAE-Cellulose Column II
GRAPH 5 DEAE-Sephadex Column I
Optical Density
Fraction Number
0
2
4
6
20
40
60
80
100
120
140
160
10
30

(ii) Column DC-II (15.0 x 1.1 cm.) was in the phosphate form buffered at pH 5.9. Elution was carried out gradientwise in two stages:-

1. 0.01 molar NaH_2PO_4/Na_2HPO_4 (150 ml.)
   $\longrightarrow$ 0.3 molar NaH_2PO_4/Na_2HPO_4 (150 ml.)
2. 0.3 molar NaH_2PO_4/Na_2HPO_4 (200 ml.)
   $\longrightarrow$ 0.3 molar NaOH (200 ml.)

(iii) Column DS-I (25.0 x 1.2 cm.) was in the chloride form. Elution was carried out gradientwise in two stages:-

1. 0.01 molar NaCl (100 ml.) $\longrightarrow$ 0.4 molar NaCl (100 ml.)
2. 0.4 molar NaCl (100 ml.) $\longrightarrow$ 3.0 molar NaCl (100 ml.)

In each case, polysaccharide (A_1) (20-30 mg.) was applied as a 1% solution to the column. Fractions were collected in 5 ml. volumes, in tubes, every 5-10 minutes. Aliquots of each tube were analysed for carbohydrate content with the phenol-concentrated sulphuric acid reagent,[81] and optical densities were measured in an EEL colourimeter. These optical densities were used to construct the accompanying elution diagrams. (Graphs 3, 4 and 5).

Tube fractions were combined as shown (table 16), concentrated to small volumes and isolated as freeze-dried solids after dialysis. Table 16 (facing page 97) also shows the proportions of sugars found in each fraction. (visual chromatographic examination of acid hydrolysates).

A small additional amount of galactose/arabinose rich

Column	Tube No	Fraction	Sugars on Hydrolysis
DC-I	2-10	1	xylose (S), glucose (S)
	11-135	2	galactose (S), arabinose (S), xylose (M), glucose (W), rhamnose (W)
	136-180	3	galactose (S), arabinose (S), xylose (M), glucose (W), rhamnose (W)
DC-II	2-14	1	xylose (S), glucose (S)
	15-70	2	galactose (S), arabinose (S), xylose (M), glucose (W), rhamnose (W)
	71-160	3	galactose (S), arabinose (S), xylose (M), glucose (W), rhamnose (W)
DS-I	1-10	1	xylose (S), glucose (S)
	11-50	2	galactose (S), arabinose (S), xylose (M), glucose (W), rhamnose (W)
	51-80	3	galactose (S), arabinose (S), xylose (M), glucose (W), rhamnose (W)

table 16

S main component
M medium component
W minor component

polymer was obtained from DC-I by washing with sodium hydroxide (0.5 molar). This material, on hydrolysis, contained an increased proportion of glucose, and it is highly probable that this arose by degradation of the DEAE-cellulose by the alkali. This procedure was therefore omitted in future experiments. Fraction 3 from DC-II also contained slightly greater quantities of glucose, probably for the same reason.

Expt. 21 Attempted Isolation of a Xylose-Rich Fraction from Polysaccharide (A_1)

(a) A solution of polysaccharide (A_1) (300 mg.; 1%) was applied to a second column of DEAE-sephadex (DS-II; 20.0 x 1.2 cm.). Elution was carried out initially with sodium chloride (0.001 molar) until tube fractions contained no carbohydrate material. (20 tubes; 5 ml./10 mins.). These tubes were combined as shown (table 17) to give two fractions. A third fraction was obtained by stripping the column with sodium chloride solution. (3.0 molar; 100 ml.). Polysaccharide fractions were isolated by freeze-drying after dialysis.

Fraction	Tube No	Weight (mg.)
1	1-6	120
2	7-20	80
3	-	60

table 17

Acidic hydrolysis and visual chromatographic examination of the fractions showed that they each contained the same sugars - galactose, arabinose and xylose, together with smaller quantities of glucose and rhamnose.

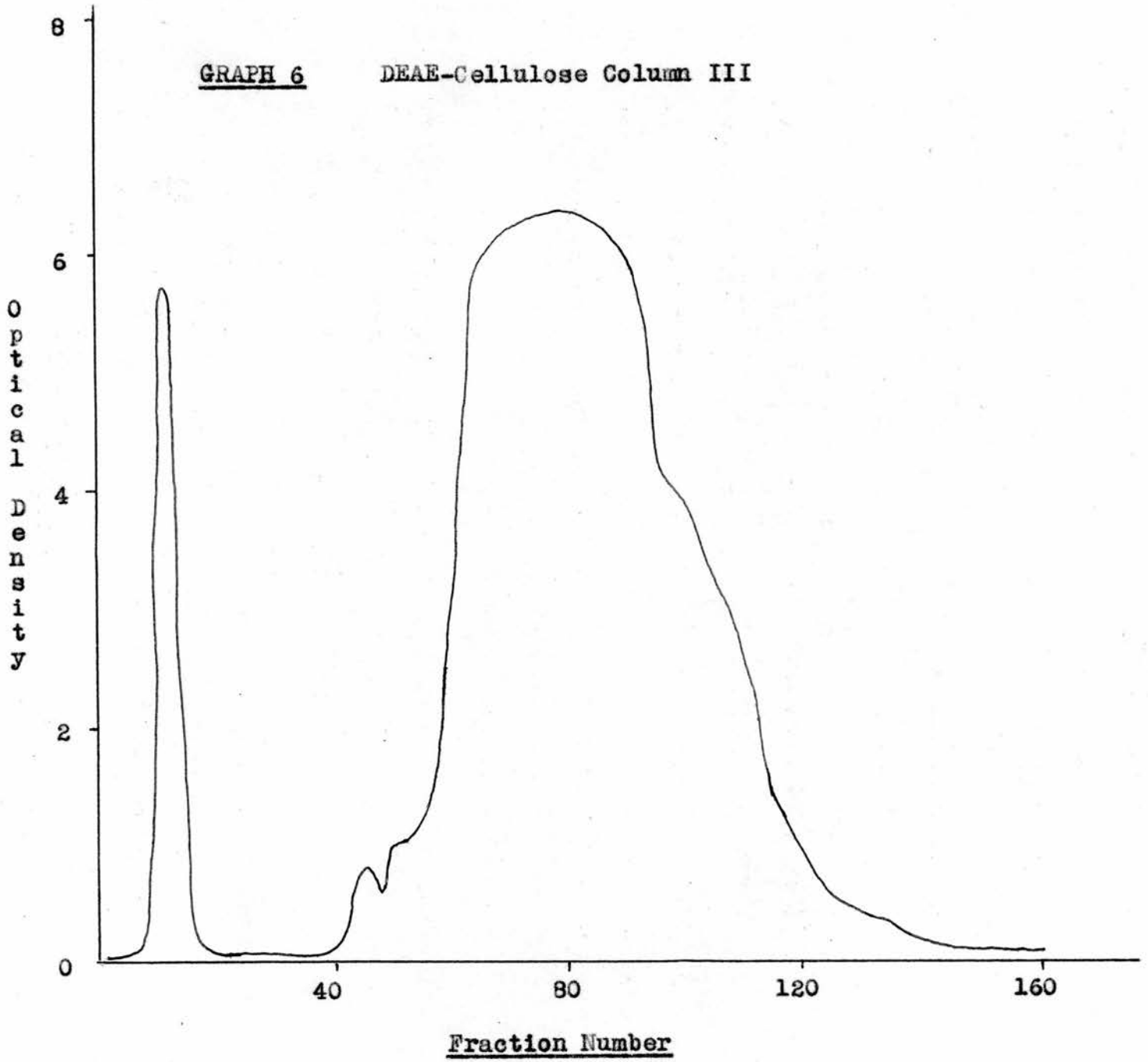
(b) Fraction 1 (110 mg.), from DS-II, was dissolved in water (10 ml.) and applied to a third DEAE-sephadex column. (DS-III; 22.0 x 1.2 cm.).

The column was eluted with sodium chloride (0.01 molar) until fractions were carbohydrate-free.(18 tubes; 5 ml./5-10 mins.). These tubes were combined as shown in table 18 to give three sub-fractions. A fourth fraction was obtained by stripping the column with sodium chloride. (3.0 molar; 100 ml.).

Fraction	Tube No	Weight (mg.)
1	1-4	2
2	5-12	40
3	13-18	10
4	-	60

table 18

Acidic hydrolysis and visual chromatographic examination of these materials showed that Fraction 1 consisted of xylose and glucose with very little of the other sugars, whereas the remaining fractions contained all the usual sugars.


GRAPH 6 DEAE-Cellulose Column III
Optical Density
0
2
4
6
8
40
80
120
160
Fraction Number

Expt. 22 Larger-Scale Purification of Polysaccharide (A_1) on DEAE-Cellulose

Polysaccharide (A_1) (512 mg.; protein - 25.6%; SO_3 - 12.3%; carbohydrate - 43.3%) was dissolved in water (50 ml.), applied to the column (DC-III; 42.0 x 3.6 cm.) and allowed to drain in. A gradient elution was commenced immediately as follows:-

1. Water (1 litre) ⟶ 0.5 molar NaCl (1 litre)
2. 0.5 molar NaCl (1 litre) ⟶ 2.0 molar NaCl (1 litre)

Fractions were collected in 25 ml. volumes every 30 minutes. Aliquots of every second tube were analysed for carbohydrate content, and an elution diagram drawn up in the usual way. (Graph 6). Tubes were combined as shown (table 19), dialysed and finally freeze-dried. The percentage recovery was 67%, after allowance for ash and protein. The isolated polysaccharide fractions gave rise to the following sugars on acid hydrolysis (visual examination).

Polysaccharide	Tube No	Wt.(mg)	Gal	Arab	Gluc	Xy	Rh
B_1	9-15	8.0	trace	trace	S	S	-
C_1	16-58	30.6	S	S	W-M	M	W
D_1	59-115	231.8	S	S	trace	M	W
E_1	116-160	38.6	S	S	trace	M	W

table 19

S major component
M medium component
W minor component

A second fractionation of polysaccharide (A_1) (910 mg.) on the same column (after regeneration by washing with chloride solutions), gave a similar though less pronounced separation, from which the percentage recovery was 46%.

The analogous polysaccharide fractions C_2 (67.9 mg.), D_2 (213.8 mg.) and E_2 (93.4 mg.) gave similar proportions of monosaccharides, on acid hydrolysis, as the above fractions from DC-III (table 19). Comparable fractions from the two separations were therefore combined for subsequent investigations, and these will be hereafter referred to as polysaccharides (C), (D) and (E).

Time did not permit further examination of polysaccharide (B).

The other polysaccharide fractions had the properties shown in table 20.

Polysaccharide	$[\alpha]_D$	Sugar(%)	SO_3(%)	Protein(%)	Ash(%)
(C)	$+59^\circ$	52.1	11.3	-	-
(D)	$+66^\circ$	49.1	13.3	16.9	9.4
(E)	$+50^\circ$*	-	14.0	-	-

*cloudy solution

table 20

None of these fractions gave a positive reaction for 3,6-anhydrogalactose. (cf. page 90).

Since polysaccharide (D) still contained a substantial protein impurity, further purification studies of polysaccharide (A_1) were made. Experiments with polysaccharides (C), (D) and (E) will be described later. (Expts. 24-28).

Expt. 23 — Further Attempts to Purify Polysaccharide (A_1)

(a) Treatment with Decolourising Charcoal and SE (Sulphoethyl)-Sephadex

Polysaccharide (A_1) (910 mg.) was dissolved in water (200 ml.). The solution was warmed at 90°C with decolourising charcoal (May and Baker; 2 g.) for 5 minutes, before filtering through a charcoal pad. The treatment was repeated.

The pale yellow solution was made 0.02 molar with sodium acetate, adjusted to pH 4.6 and passed down a column of SE (sulphoethyl)-sephadex. (10.0 x 2.9 cm.).

The SE-sephadex was the Pharmacia C_{50} grade. It was pre-treated in exactly the same manner as described for DEAE-cellulose (page 95), and was equilibrated with sodium acetate buffer (0.02 molar; pH 4.6) before use.

After application of the polysaccharide, the column was washed with sodium acetate solution (0.02 molar; pH 4.6) until the effluent gave a negative phenol-concentrated sulphuric acid reaction.[81] The total effluent was dialysed and freeze-dried to a nearly white solid. (212 mg.; 30%). This was known as polysaccharide (F) and it had the properties shown in table 21.

Sugar(%)	$[\alpha]_D$	SO_3(%)	Protein(%)	Ash(%)
49.6	+73°	17.0	14.4	8.9

table 21

In addition, polysaccharide (F) gave rise to the molar proportions of sugars shown in table 22.

Monosaccharide	Galactose	Arabinose	Xylose
Molar Proportions	2.0	2.4	1.0

table 22

(b) Treatment with Sulphoethyl-Sephadex

Pre-treated SE-sephadex (page 95) was equilibrated with citric acid-sodium monohydrogen phosphate buffer (0.5 molar; pH 3.4), filtered and washed thoroughly with the same buffer of lower concentration. (0.02 molar). The material was finally packed as a slurry in this solution to give a column 10.0 x 1.5 cm.

A buffered solution (0.02 molar; pH 3.4) of polysaccharide (A_1) (100 mg.) was percolated into the column and then eluted with buffer of the same concentration until the effluent gave a negative test for carbohydrate.[81] It was noticed that a large proportion of the brown colouring in the solution was adsorbed at the top of the column.

The effluent was dialysed, concentrated and freeze-dried. The product (72 mg.) had a somewhat higher protein content (28.8%) than polysaccharide (A_1).

(c) Treatment with CM (carboxymethyl)-Cellulose

The experimental procedure was the same as described for (b), except that sodium acetate buffers (pH 6; 0.5 and 0.02 molar) were used. The CM-cellulose was the Whatman

derivative.

The recovered freeze-dried substance (65 mg.) had a protein content of 19.4%. It gave rise to the usual proportions of sugars on acid hydrolysis, as did the recovered material from Expt. 23(b).

(d) Treatment with Phenol [143]

A 2% aqueous solution of polysaccharide (A_1) was adjusted to 45% (w/v) concentration with phenol, warmed to 70°C, shaken gently for 30 minutes, then set aside at 2°C.

This mixture did not separate into two layers as expected, even on prolonged standing. An emulsion was formed which was not dispersed by any conventional means, such as addition of salts, butyric acid or filtering through Kieselguhr.

No polysaccharide was recovered from this experiment.

Expt. 24 Examination of Polysaccharide (C)

Polysaccharide (C) had the properties shown in table 20 (page 100). It gave rise to the molar proportions of sugars [142] shown in table 23 on acid hydrolysis.

Monosaccharide	Galactose	Arabinose	Xylose
Molar Proportions	2.1	2.6	1.0

table 23

It also contained a greater proportion of glucose than the related polysaccharides (D) and (E). (table 19, page 99). This was estimated to be 2.5%, according to the method of

White and Subers.[144] (by Mrs. M. Kerr). The nature of this glucose was investigated in the following experiments.

(a) Treatment with Salivary ∝-Amylase

Polysaccharide (C) (14.2 mg.) was dissolved in water (5 ml.) containing sodium chloride (1 mg.) and salivary ∝-amylase (20 mg.). The mixture was incubated at 37°C for 24 hours and the residual polymer isolated by freeze-drying after dialysis. This material (9.6 mg.) still contained glucose (1.7%).[144]

(b) Treatment with Sodium Metaperiodate

Polysaccharide (C) (17.5 mg.) was dissolved in water (5 ml.) containing sodium metaperiodate (0.107 g.; i.e. 0.1 molar). The mixture was left in the dark for 72 hours at room temperature.

Excess periodate was reduced with ethylene glycol, and the oxidised polymer was treated with potassium borohydride (20 mg.) at room temperature. The residual polymer (12.9 mg.) was isolated by freeze-drying after dialysis. This material also contained a residual quantity of glucose (0.5%).

Expt. 25 Examination of Polysaccharide (D)

Polysaccharide (D) had the properties shown in table 20. (page 100). The molar proportions of monosaccharides were estimated by the Wilson method,[142] and the values are shown in table 24.

Monosaccharide	Galactose	Arabinose	Xylose
Molar Proportions	2.4	2.7	1.0

table 24

(a) Treatment with Decolourising Charcoal

Polysaccharide (D) (170 mg.) was heated in water (20 ml.) with decolourising charcoal (May and Baker; 0.4 g.) as described previously for polysaccharide (A_1). (Expt. 23a; page 101).

The residual polysaccharide (G) was isolated as a pure white solid by freeze-drying the dialysed solution. (67.7 mg.; 44%). Polysaccharide (G) had the properties shown in table 25.

Sugar(%)	$[\alpha]_D$	SO_3(%)	Protein(%)	Ash(%)
52.2	$+80^\circ$	19.1	8.1	10.0

table 25

In addition, an acid hydrolysate of polysaccharide (G) contained the molar proportions of sugars shown in table 26.

Monosaccharide	Galactose	Arabinose	Xylose
Molar Proportions	2.6	2.9	1.0

table 26

A test for 3,6-anhydrogalactose (cf. page 90) was negative.

Expt. 26 Treatment of Polysaccharide (G) with Alkali

Polysaccharide (G) (30.0 mg.) was dissolved in water (5 ml.) containing potassium borohydride (30 mg.), and left overnight at room temperature. A further portion of potassium borohydride (30 mg.) and sodium hydroxide (2N; 5 ml.) were added, and the

solution was heated at 80°C 2°C for 7 hours. Additions of potassium borohydride (1-2 mg.) were made every hour.

The solution was cooled, dialysed against frequently changed distilled water, concentrated and freeze-dried to give a polysaccharide (H). (24.2 mg.; 81%). It had the properties shown in table 27.

Sugar(%)	$[\alpha]_D$	SO_3(%)	3,6-Anhydrogalactose(%)
59.9	+65°	13.8	ca. 0.2

table 27

In addition, an acid hydrolysate of polysaccharide (H) contained the molar proportions of sugars shown in table 28.[142]

Monosaccharide	Galactose	Arabinose	Xylose
Molar Proportions	2.3	2.0	1.0

table 28

Expt. 27 Examination of Polysaccharide (E)

Polysaccharide (E) was very similar to polysaccharide (D) in sulphate content (table 20; page 100) and gave rise to similar proportions of sugars on acid hydrolysis.[142] (table 29).

Monosaccharide	Galactose	Arabinose	Xylose
Molar Proportions	2.5	2.8	1.0

table 29

cf. Polysaccharide (D) (table 24; page 104).

Application of Smith Degradation Technique[145]

Polysaccharide (E) (76.4 mg.) was suspended in sodium metaperiodate solution (40 ml.; 0.015 molar) and shaken in the dark at room temperature. The reduction of periodate was determined, at intervals, by the method of Aspinall and Ferrier.[87] The following data were obtained. (table 30).

Time (hours)	2	24	49	73	122	145	168
Moles periodate/ anhydrohexose unit	0.074	0.177	0.232	0.265	0.299	0.327	0.328

table 30

The reaction was stopped by the addition of ethylene glycol, and the oxopolyaldehyde was reduced with potassium borohydride. The derived polyalcohol (49.1 mg.) was dissolved in water (6.0 ml.), and a portion of this solution (1.0 ml.) was removed for complete acid hydrolysis. Chromatographic examination of the hydrolysate showed the presence of galactose and arabinose, together with traces of xylose and rhamnose. Development of chromatograms with alkaline silver nitrate also revealed substances with chromatographic mobilities comparable with glycerol and xylose/erythritol/threitol. (solvent 1). The latter substance gave a stronger spot than that expected for a trace quantity of xylose.

The remainder of the polyalcohol solution (5.0 ml.) was diluted with sulphuric acid (2N; 5.0 ml.) and transferred to a polarimeter tube at room temperature. Initially the scale-reading was 0.15, corresponding to $[\alpha]_D$ +38°. After 30

minutes, the solution had become too cloudy to see through, due to the formation of a fine precipitate. Hydrolysis was continued, under these conditions, for 6 hours, and then the residual polymer was isolated by centrifugation, following the addition of ethanol (200 ml.).

The precipitate was washed thoroughly with ethanol, dissolved in dilute ammonia (0.05 molar), dialysed and freeze-dried. The recovered degraded polysaccharide (J) (18.2 mg.) gave rise to the molar proportions[142] of sugars shown in table 31 on acid hydrolysis.

Monosaccharide	Galactose	Arabinose
Molar Proportions	1.2	1.0

table 31

The alcoholic supernatant solution was de-ionised with Amberlite IR 120(H) and IR 45B(OH) ion-exchange resins and concentrated to a syrup. This contained at least two non-reducing substances of comparable mobility to glycerol and erythritol/threitol. (solvents 1 and 2; reagent 3.).

Expt. 28 Micromethylation of Polysaccharides (E) and (J)

Polysaccharide (1.5-2.5 mg.) was dissolved in dimethylsulphoxide (0.10 ml.) with warming. The solution was cooled to 0°C and diluted with dimethylformamide (0.10 ml.). Barium hydroxide octahydrate (0.1 g.) and dimethylsulphate (0.07 ml.) were added and the mixture shaken in an ice-bath

After two hours, the temperature was allowed to rise, and shaking was continued at room temperature for 40 hours.

The reaction mixture was shaken with concentrated ammonia (0.05 ml.) for one hour and extracted with chloroform. (3 x 20 ml.). The chloroform layers were taken to dryness and refluxed with dry methanolic hydrogen chloride (3%; 2 ml.) in sealed tubes for 12 hours.

Gas-liquid chromatographic examination[85] indicated that both mixtures were too complex to give an unambiguous identification of the constituents.

2. Desulphation Studies

Expt. 29 — Treatment of Polysaccharide (A_1)[146] with Methanolic Hydrogen Chloride

Polysaccharide (A_1) (203 mg.) was shaken with dry methanolic hydrogen chloride (40 ml.; 1%) at room temperature. After 24 hours, the residual polymer (ca.120 mg.) was recovered by centrifugation, and a portion (20 mg.) taken for acid hydrolysis (and chromatographic examination) and estimation of sulphate. A portion (5 ml.) of the supernatant was also hydrolysed with sulphuric acid and examined chromatographically. The remainder of the polymer and the supernatant solution were recombined and shaken for a further 24 hours.

The residual polymer, after this time, was isolated as before, and a portion (20 mg.) of it and the supernatant examined in the same way as previously. The remainder of

Time(hours)	Sample	Gal	Arab	Xy	Gluc	Rh	SO_3(%)
24	Supernatant	S	W	M	W	-	-
	Residue	M	S	W	W	W	12.1
48	Supernatant	W	S	W	W	W	-
	Residue	S	S	M	W	W	3.6
96	Supernatant	S	S	M	W	W	-
	Residue	S	S	M	W	W	2.3

table 32

S major component

M medium component

W minor component

this degraded material (ca. 80 mg.) was then shaken with a fresh portion of methanolic hydrogen chloride (40 ml.; 1%) for a further 24 hours. After this time, the residual polymer (ca. 40 mg.) and the supernatant were separated and examined as before. The data are given in table 32 on the facing page.

The final residual polysaccharide (K) obtained after the treatment for 96 hours gave rise to the molar proportions of sugars shown in table 33 on acid hydrolysis.

Monosaccharide	Galactose	Arabinose	Xylose
Molar Proportions	2.9	3.8	1.0

table 33

Certain interesting changes in the infra-red spectrum were also observed as sulphate was removed from polysaccharide (A_1). Initially, polysaccharide (A_1) showed broad bands at 1210-1250 $cm.^{-1}$ and at 810-840 $cm.^{-1}$ (see pages 24 and 28).

The spectrum of the residue after 24 hours showed little change from that of the original polysaccharide (A_1), whereas the spectra of both the 48 and 96 hour residues showed striking modifications. In each case, the general sulphate absorption band at 1210-1250 $cm.^{-1}$ had become very much smaller, while the second band at 810-840 $cm.^{-1}$ had sharpened considerably to a peak at 819 $cm.^{-1}$

A similar series of desulphation experiments was carried out with 0.1% methanolic hydrogen chloride. The residual

polysaccharide after treatment for 96 hours (ca. 25%) contained 7.1% of ester sulphate. The infra-red spectrum of this substance showed very little difference from that of the original polysaccharide (A_1).

Examination of the infra-red absorptions of the Chaetomorpha polysaccharides (A_2) and (A_3) showed that, in addition to the general absorption about 1240 cm.$^{-1}$, they possessed a specific band at 825-835 cm.$^{-1}$ and very little absorption at 820 cm.$^{-1}$

Expt. 30 **Oxidation of Polysaccharide (K) with Sodium Metaperiodate**

Polysaccharide (K) (Expt. 29; page 110; 4.5 mg.) was treated in the dark, at room temperature, with sodium metaperiodate solution (0.015 molar; 2 ml.). At intervals, the reduction of periodate was measured by the method of Aspinall and Ferrier.[87] The following data were obtained. (table 34).

Time(hours)	20	48	72	96	120
Moles periodate/ anhydrohexose unit	0.404	0.443	0.483	0.495	0.496

table 34

The reaction was stopped by addition of ethylene glycol and potassium borohydride (10 mg.) was added. After 24 hours at room temperature, the residual polysaccharide was hydrolysed. The hydrolysate was neutralised, de-ionised and concentrated

to a syrup. Estimation of the molar proportions[142] of galactose and arabinose (the sole uncleaved monosaccharides) gave the values shown in table 35.

Monosaccharide	Galactose	Arabinose
Molar Proportions	1.9	1.0

table 35

Expt. 31 Treatment of Polysaccharide (A_1) with Diazomethane[147]

A solution of polysaccharide (A_1) (1.0g; 1%) was converted to the free acid form with Amberlite IR 120(H) ion-exchange resin, concentrated to a small volume (20 ml.) and diluted with methanol to the point of precipitation.

A chilled solution of diazomethane (prepared by alkaline distillation of p-tolyl sulphonylmethyl nitrosamide) was added in portions, with shaking, to the chilled polysaccharide solution, until the upper ethereal layer remained yellow.

The mixture was shaken vigorously at 2°C for 20 hours, diluted with water, concentrated to remove methanol and ether, and freeze-dried to give polysaccharide (I) (0.8 g.).

An aqueous solution of this polysaccharide was still acid to litmus, indicating that esterification was not complete.

Expt. 32 Desulphation of Polysaccharide (I)

(a) Reductive Fission with Lithium Aluminium Hydride

Polysaccharide (I) (315 mg.) was refluxed overnight in dry dioxane (80 ml.) containing lithium aluminium hydride. (0.4 g.).

Excess lithium aluminium hydride was removed with ethyl acetate, and hydrochloric acid was added to pH 5. The mixture was filtered, washed with water, and the filtrate plus washings freeze-dried to a brown solid (13 mg.), after dialysis.

The material contained 2.3% sulphate.

(b) Treatment of Polysaccharide (I) with Methanolic Hydrogen Chloride

Polysaccharide (I) (208 mg.) was shaken overnight, at room temperature, with methanolic hydrogen chloride. (40 ml.;1%).

The mixture was neutralised with methanolic ammonia, concentrated to remove methanol, and freeze-dried.

The residual material (52 mg.) contained 2.3% sulphate.

Expt. 33 Desulphation of Polysaccharide (A_1) with Sodium Hydroxide

Polysaccharide (A_1) (100.5 mg.) was treated in the manner already described for polysaccharide (G). (page 105; Expt. 26).

The residual polymer (55 mg.; 55%) contained sulphate (9.3%), and on acid hydrolysis, gave rise to a trace quantity of a new substance, which gave a pink colour with aniline oxalate, as well as the usual monosaccharides. This new substance had a chromatographic mobility comparable with rhamnose in solvents 1 and 2.

Expt. 34 Treatment of Polysaccharides (A_1), (A_2) and (A_3) with Sodium Methoxide

Polysaccharide (ca. 200 mg.), in water (10 ml.) containing potassium borohydride (20 mg.), was left at room temperature overnight, then dialysed and freeze-dried. To the freeze-dried, reduced materials (dried further in vacuo for 48 hours) were added dry methanol (22 ml.), potassium borohydride (0.1 g.) and sodium metal, (0.5 g.), in portions.

The mixtures were refluxed with stirring for 48 hours, and the residual polysaccharides (ca. 100 mg.) were recovered by filtering and washing with methanol.

Chromatographic examination of acid hydrolysates of each of these materials gave the same pattern of sugars, including two new substances, (P) and (Q).

Substance (P) (R_{gal} 1.82 in solvent 1; R_{gal} 3.92 in solvent 2) was identical with 2-O-methyl xylose in chromatographic mobility, and colour given with aniline oxalate. (reddish-black).

Substance (Q) (R_{gal} 1.60 in solvent 1; R_{gal} 3.49 in solvent 2) gave a bright red colour with aniline oxalate. It had a chromatographic mobility distinctly different from 2-O-methyl arabinose, 2-O-methyl xylose and 3-O-methyl xylose in solvents 1 and 2.

When paper chromatograms were treated with triphenyl-tetrazolium hydroxide[88], substance (Q) gave a deep red colour, while substance (P) gave a slight positive reaction. Under the same conditions, authentic 2-O-methyl xylose gave hardly any perceptible colour. This indicated that the main portion

of substance (P) was probably substituted at C_2, but that it also contained an impurity which had the C_2 hydroxyl free.

This was confirmed when the syrups were examined in solvent 4, when a third substance (R) (R_{gal}10.7) separated from (P) (R_{gal} 9.8) and (Q) (R_{gal} 6.1). Substance (R) had a chromatographic mobility identical with 3-O-methyl xylose, and like 3-O-methyl xylose, (R) gave a bright red colour with aniline oxalate and a positive reaction with triphenyltetrazolium hydroxide.[88] Substance (P) gave no perceptible colour with the triphenyltetrazolium hydroxide reagent after chromatography in solvent 4.

It perhaps should be emphasised that the above reactions were confirmed for each of the syrups obtained from polysaccharides (A_1), (A_2) and (A_3).

Expt. 35 Large-Scale Treatment of Polysaccharide (A_1) with Sodium Methoxide

Polysaccharide (A_1) (10 g.) was dissolved in water (1 litre) containing sodium borohydride (1 g.) and left overnight at room temperature. The residual polysaccharide (7.5 g.), isolated after dialysis and freeze-drying, was dried in vacuo over phosphorus pentoxide at 60°C for 4 days, and then treated with dry methanol (400 ml.) for 2 days to remove last traces of moisture. The polysaccharide was quickly centrifuged and added to a solution of sodium methoxide (6.0 g. sodium in 250 ml. dry methanol) containing sodium borohydride. (0.5 g.).

The mixture was refluxed for 24 hours with further

additions of sodium borohydride (1 g.) in portions.

The residual polysaccharide (L) was recovered by filtration and washed with methanol (4 litres) to remove most of the inorganic material. (Yield 6 g.). Chromatographic examination of an acid hydrolysate of polysaccharide (L) (solvent 4) revealed the presence of substances (P), (Q) and (R) as before. (page 114; Expt. 34).

Further treatment of portions of polysaccharide (L) (0.1 g.) with fresh volumes of sodium methoxide for 24, 72 and 168 hours did not increase the proportions of any of the new substances.

Expt. 36 Hydrolysis of Polysaccharide (L) and Separation of Monomethyl Pentoses

Polysaccharide (L) (5.0 g.) was hydrolysed with sulphuric acid (N; 250 ml.) for 5 hours at 100°C. The hydrolysate was cooled and neutralised in the usual way to give a clear yellow syrup. (1.9 g.). The syrup was made into a paste with cellulose, water (54 ml.) and butanol (6 ml.), and applied as a slurry to a prepared cellulose column. (55 x 4 cm.).

The column was eluted with redistilled butanol, half saturated with water. Fractions (25 ml.) were collected every 30 minutes. Aliquots (4 ml.) of every second tube were concentrated to a small volume and examined by paper chromatography in solvents 1 and 4. Tubes of similar content were combined to give the following fractions:-

Fraction (L_1) (75 mg.) contained the two components, (P) and (R). (page 114; Expt. 34).

Fraction (L_2) (137 mg.) was a complex mixture of monosaccharides. In addition to substances (P), (Q) and (R), it contained rhamnose and xylose.

Expt. 37 Examination of Fraction (L_1) and Isolation of 2-O-methyl-L-xylose

Fraction (L_1) was a syrup. (75 mg.). It had $[\alpha]_D$ -24° (c. 0.75 in water), and contained two components (P) and (R) on chromatographic examination in solvent 4.

The major component (P) had a chromatographic mobility identical with that of 2-O-methyl xylose and like the latter, it did not give a colour with triphenyltetrazolium hydroxide.[88]

Component (R) was present in trace quantity. It had the same chromatographic mobility as 3-O-methyl xylose, and gave a positive reaction with triphenyltetrazolium hydroxide.[88]

(a) Demethylation[86]

A portion (ca. 10 mg.) of fraction (L_1) in dichloromethane (1 ml.) was demethylated with boron trichloride. Chromatographic examination of the residue (solvents 1, 2 and 3) showed that xylose was the sole product.

(b) Methanolysis (page 40)

A sample of fraction (L_1) (1-2 mg.) was refluxed with dry methanolic hydrogen chloride (3%) for 8 hours.

Gas-liquid chromatographic examination[85] of the methanolised mixture showed peaks, whose retention values were characteristic of the methyl glycosides of 2-O-methyl xylose. In addition, two small peaks, whose retention values were characteristic of 3-O-methyl xylose, were observed.

(c) Isolation of Crystalline 2-O-methyl-L-Xylose

The remainder of the syrup (L_1) deposited white needles from ethanol at room temperature. These were isolated, washed with ethyl acetate/ethanol (2/1) and recrystallised twice from absolute ethanol. The solid melted in the range 132-133°C and possessed the chromatographic properties of 2-O-methyl xylose. (pages 114-115). Admixture of the crystalline compound with authentic crystalline 2-O-methyl-D-xylose (mp 135°) gave a depressed melting range. An X-ray powder photograph of the crystalline substance (P) was identical with that of authentic 2-O-methyl-D-xylose, indicating that (P) was in fact the L-isomer of 2-O-methyl xylose.

The mutarotation of substance (P) was studied in a 1 dm. polarimeter tube at room temperature (c. 0.5 in water), when the following data were obtained. (table 36).

Time(mins.)	8	15	30	60	90	180	300
$[\alpha]_D$	-6.4°	-22.3°	-28.7°	-32.9°	-34.0°	-35.1°	-36.1°

table 36

The rotation was constant at -36.1° after 24 hours.

Expt. 38 Examination of Fraction (L_2)

The syrup (137 mg.) contained several components on chromatographic examination. (Expt. 36; page 117).

Substance (Q) (Expt. 34; page 114) was present as a minor component and was not readily separated from contaminating

rhamnose which had a comparable chromatographic mobility.

Successive separations on filter sheets gave a small amount (1 mg.) of apparently pure substance (Q). This gave rise to arabinose only on demethylation[86] and it had a chromatographic mobility (Expt. 34; page 114) different from authentic 2-O-methyl arabinose. It also differed from 2-O-methyl arabinose in the colour it gave with aniline oxalate (bright red as compared with reddish-black given by the 2-substituted compound), and in its positive reaction with triphenyltetrazolium hydroxide.[88]

Gas-liquid chromatographic examination of the derived methyl glycosides of substance (Q) showed two peaks whose retention values were characteristic of the methyl glycosides of 3-O-methyl arabinose.[85] This examination also indicated that (Q) was contaminated with other unknown substances.

3. Partial Fragmentation Studies

Expt. 39 Preliminary Examination of the Partial Hydrolysis Products of Polysaccharides (A_1), (A_2) and (A_3)

Samples of polysaccharides (A_1), (A_2) and (A_3) (ca. 0.1 g.) were hydrolysed (N sulphuric acid; 10 ml.) at 100°C for 1 hour, neutralised in the usual way, and the resultant solutions de-ionised with Amberlite IR 120(H) ion-exchange resin.

Ionophoretic examination (page 41) of the derived syrups showed the presence of several charged components, the pattern being identical for (A_1), (A_2) and (A_3). (table 37; page 120). Major proportions of neutral sugars were also present.

Component	Colour with Aniline Oxalate	M_G Value
1	brown	0.70
2	pink	0.80
3	brown	1.00
4	pink	1.18
5	trace — yellow	1.30
6	trace — pink	1.50

table 37

M_G values are expressed relative to the migration of authentic galactose-6-sulphate.

Thus, $M_GX = \frac{\text{migration distance of compound X}}{\text{migration distance of galactose-6-sulphate}}$

The most satisfactory chromatography of these acidic syrups was achieved in solvent 1. In addition to the usual neutral sugars, slower sugars were also evident. The principal of these had R_{gal} 0.55 (identical with galactose-6-sulphate, and different from galactose-4-sulphate), and R_{gal} 0.83. These substances gave brown and pink colours with aniline oxalate respectively. Each of the syrups from (A_1), (A_2) and (A_3) were applied to squares of Whatman 3MM paper and subjected to ionophoresis under the same conditions as before. The charged sugars were located, eluted from the papers and concentrated to syrups. Ionophoretic examination of these syrups showed that neutral sugars were absent.

Portions of each syrup were accordingly hydrolysed (0.5N HCl) at 100°C for 6 hours, and the hydrolysates

re-examined by ionophoresis and paper chromatography. (solvent 1). The former experiment showed that no charged fragments had survived the hydrolysis conditions, while the paper chromatographic examination indicated that the hydrolysates contained galactose and arabinose. Furthermore, the remainder of the hydrolysed solutions gave a white precipitate (thought to be barium sulphate) on addition of barium chloride solution.

It was therefore considered that the original hydrolytic conditions (page 119) were probably suitable for the release of carbohydrate sulphate derivatives. Further trial experiments showed that the greatest proportions of components 3 and 4 (table 37; page 120), which were presumed to be sulphated monosaccharides, were obtainable when the polysaccharides were hydrolysed (N sulphuric acid) for 1.5 hours at 100°C.

Expt. 40 — Acidic Hydrolysis of Polysaccharide (A_1) and Separation into Acidic (S) and Neutral (R) Fractions

Polysaccharide (A_1) (10 g.) in sulphuric acid (N; 250 ml.) was heated for 1.5 hours at 100°C. The hydrolysate was cooled, neutralised with solid barium carbonate, filtered, de-ionised with Amberlite IR 120(H) ion-exchange resin and concentrated carefully to a small volume. (50 ml.). This solution was percolated through a column (12 x 2.7 cm.) of Amberlite IR 400A (acetate form) ion-exchange resin.

The column was then washed with water until the effluent gave a negative test for carbohydrate.[81] This effluent, which contained neutral sugars, was concentrated to a syrup (R) (2.7 g.)

and set aside at 0°C.

The column was next washed with sulphuric acid (0.5N; 2 litres) to displace acidic fragments from the ion-exchange resin. The acidic effluent was cooled (0°C), neutralised with solid barium carbonate, filtered, de-ionised with Amberlite IR 120(H) resin and concentrated on a rotary film evaporator, with additions of water to remove acetic acid. When the distillate was neutral, the solution was neutralised (pH 8) with dilute ammonium hydroxide and freeze-dried to a white solid (S).

Ionophoretic examination of a solution of (S) revealed that the same components as listed in table 37 (page 120) were present, and that neutral materials had been successfully removed.

Expt. 41 Preliminary Separation of Fraction (S) on a Charcoal Column

Fraction (S) was dissolved in water and applied to a column of charcoal. (May and Baker decolourising grade; 3 x 9 cm.). The column was washed with distilled water and fractions (25 ml.) collected every hour. Aliquots (1 ml.) of tube fractions were removed, concentrated and examined by ionophoresis. (page 41). Tubes containing similar components were then combined to give three main fractions.

These were each treated with Amberlite IR 120(H) ion-exchange resin and de-salted by shaking with a solution of tri-N-octylamine (5%) in chloroform. (4 x 20 ml.). The aqueous solutions were finally washed with chloroform,

neutralised (pH 8) with ammonium hydroxide, concentrated and freeze-dried. The following fractions were obtained:-

Fraction (S_1) (582 mg.) contained two components. The major one, compound (S_B), had M_G 1.18, R_{gal} 0.83 (solvent 1) and gave a pink colour with aniline oxalate. The minor component, compound S_A, had M_G 1.00 and R_{gal} 0.55 (solvent 1), comparable with authentic galactose-6-sulphate, and like the latter, it gave a brown colour with aniline oxalate.

Fraction (S_2) (180 mg.) consisted mainly of a substance with the same properties as the minor component (S_A) of fraction (S_1). It also contained a small amount of compound (S_B).

Fraction (S_3) (50 mg.) contained substances with M_G values of 0.70 and 0.80, which gave brown and pink colours with aniline oxalate respectively, as well as compounds (S_A) and (S_B).

Expt. 42 Isolation of Compounds (S_A) and (S_B) from Acidic Fractions (S_1) and (S_2)

Fractions (S_1) and (S_2) (Expt. 41) were dissolved in water, applied to 3MM filter paper sheets and eluted in solvent 1 for 24 hours. Sugars were located by means of side-strips, appropriate sections of paper cut out, eluted with water and concentrated to syrups.

Successive separations in this way produced specimens of chromatographically (solvents 1 and 3), and ionophoretically homogeneous compounds (S_A) (95 mg.) and (S_B) (56.5 mg.), as their ammonium salts.

Expt. 43 Characterisation of Compounds (S_A) and (S_B) as Galactose-6-Ammonium Sulphate and Arabinose-3-Ammonium Sulphate

Compounds (S_A) and (S_B) had $[\alpha]_D$ +51° (c. 1.3) and $[\alpha]_D$ +75° (c. 0.6) respectively, calculated as the ammonium salts of hexose and pentose monosulphates from sugar concentrations found by means of the phenol-concentrated sulphuric acid reagent.[81]

Standard solutions of compounds (S_A) (1.286 mg./ml.) and (S_B) (1.687 mg./ml.) were prepared and used for the following experiments:-

(a) Degree of Polymerisation[82]

Aliquots (0.1 ml.) of each solution were used to measure the degree of polymerisation by the Timell method. (see page 38).

The values obtained for compounds (S_A) and (S_B) were 1.03 and 1.02 respectively.

(b) Estimation of the Molar Ratio of Monosaccharide and Sulphate

Samples of (S_A) and (S_B) (0.500 ml.) containing sodium chloride (1 mg.) were taken to dryness, and digested in sealed tubes with concentrated nitric acid. (0.25 ml.).

The amounts of sulphate released were measured, in the usual way, by the method of Jones and Letham,[79] (see page 36), and monosaccharides were determined with the phenol-concentrated sulphuric acid reagent.[81]

The molar proportions of monosaccharide and sulphate (SO_4) obtained, are given in table 38. (page 125).

	Molar Proportions		
Compound	Galactose	Arabinose	Sulphate
(S_A)	1.00	-	1.15
(S_B)	-	1.00	1.09

table 38

(c) Oxidation of Compounds (S_A) and (S_B) with Sodium Metaperiodate

(i) Reduction of Periodate

Aliquots of (S_A) and (S_B) (0.500 ml.) were diluted with sodium metaperiodate solution (0.500 ml.; 0.03 molar) and stored in the dark at room temperature. Aliquots (0.1 ml.) of each mixture were withdrawn at intervals, and the reduction of periodate was determined, in the usual manner, by the method of Aspinall and Ferrier[87]. The results are shown in table 39.

	Reduction of IO_4^- per Mole (S_A) and (S_B)	
Time (hours)	(S_A)	(S_B)
0.75	3.08	0.54
2.5	3.56	1.07
4.0	3.36	1.34
24	3.67	2.02
96	3.79	2.95
192	-	3.30

table 39

(ii) Formaldehyde Released from (S_A) and (S_B)[149] during Periodate Oxidation

Formaldehyde was estimated by means of chromotropic acid reagent, prepared by dissolving the sodium salt of chromotropic acid (1 g.) in water (100 ml.), filtering through glass wool and making up to 500 ml. with sulphuric acid. (12.5 molar).

Samples of (S_A) and (S_B) (0.05-0.075 ml.) and authentic glucose-3-ammonium sulphate (116-174 μg.) (kindly given by Drs. D. A. Rees and J. R. Turvey) were oxidised with sodium metaperiodate solution (0.015 molar) in a total volume of 0.200 ml., (a) in unbuffered solution, and (b) in sodium bicarbonate buffer. (0.05 molar; pH 7.5).

Formaldehyde (10-20 μg.) was determined by treating the samples with freshly prepared aqueous sodium sulphite (1 ml.; 5%), chromotropic acid reagent (10 ml.) and heating in stoppered tubes for 30 minutes, in a boiling water bath.

The tubes were cooled, diluted with thiourea (2 ml.; 4.6%) and the optical densities of the contents were read at 570 mμ. in a Unicam SP 600 spectrophotometer, against a blank prepared in the same way.

Standard quantities of formaldehyde were obtained by oxidising authentic purified erythritol for 1 hour under the same conditions. The amounts of formaldehyde released per mole of (S_A) and (S_B), in unbuffered and buffered conditions, are given in tables 40a and 40b. (page 127).

	(S_A)	(S_B)	Glucose-3-Sulphate
Moles H.CHO/Mole (S_A) and (S_B) at 96 hours	None	0.48	0.38

table 40a

Moles H.CHO/Mole Sugar at pH 7.5

Time (hours)	(S_A)	(S_B)	Glucose-3-Sulphate
1	-	trace	trace
3	-	0.63	0.60
24	-	0.79	0.81

table 40b

(d) Methylation of Compounds (S_A) and (S_B)

Samples of (S_A) and (S_B) (1-2 mg.) were methylated according to Bishop and Perila's modification[84] of the Kuhn procedure. (page 39). The methylated products were methanolised and examined by gas-liquid chromatography.[85]

The products from (S_A) showed peaks, characteristic of methyl 2,3,4-tri-O-methyl galactosides, while the products from (S_B) contained methyl 2,4- and 2,5-di-O-methyl arabinosides. This confirmed that, in each respective case, the C_6 and C_3 hydroxyls were blocked by sulphate groupings.

(e) Rate of Hydrolysis of Ester Sulphate[51]

Samples of compounds (S_A) and (S_B) in hydrochloric acid (0.250 ml.; 0.250N), containing about 50μg. of esterified sulphate, were sealed in pyrex tubes (3" x $\frac{3}{4}$") and heated in a boiling water-bath for varying periods. After cooling, centrifuging and opening, the contents of each tube were exactly neutralised with sodium hydroxide (2.5N; 0.025 ml.), and a trace of cetavlon and CAD reagent (0.250 ml.; page 36) added. After mixing, allowing to stand for several hours and clarification of the mixtures on the centrifuge,

portions (0.1 ml.) of the supernatants were diluted with hydrochloric acid (0.1N; 25 ml.), and the optical densities of the solutions were measured at 254 mμ., in the usual way.

Since all known carbohydrate sulphate esters exhibit first order rate kinetics on acid hydrolysis[50], the general first order rate equation (i) will be valid, i.e. $\log \frac{a}{a-x} = k't$ (i) where a is the initial concentration of reactant, and c is the concentration after time t hours.

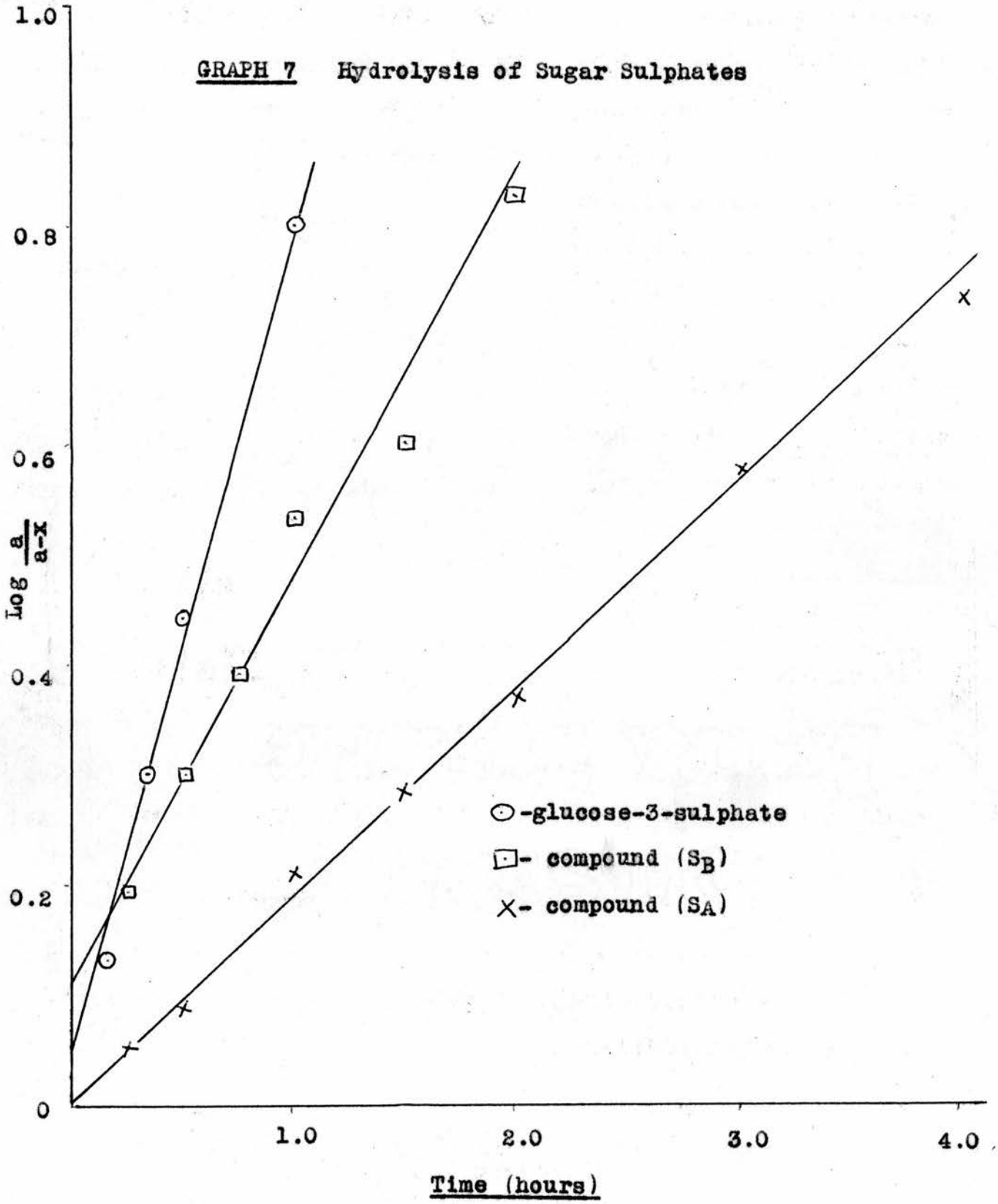
The rate constant k' is given by the gradient of the straight line obtained by plotting $\log \frac{a}{a-x}$ against time. An important derivative from equation (i) is the term 'half-life' ($t_{\frac{1}{2}}$). This is the time required to reduce the concentration of reactant to half its initial value. At this time, $x = a/2$, and by substitution of this value in equation (i), we find that $t_{\frac{1}{2}} = \frac{\log 2}{\text{slope}}$.

In the present experiment, values of $\log \frac{a}{a-x}$ were obtained from the expression,

$$\frac{a}{a-x} = \frac{E_{blank} - E_{24}}{E_t - E_{24}}$$

where E_{24} is the optical density at 24 hours, that is after total hydrolysis of the ester sulphate, and E_t is the optical density after hydrolysis for t hours.

The experimental data are given in tables 41a, 41b and 41c (page 129), and the graphical plots are shown in graph 7. (facing page 129). The half-life ($t_{\frac{1}{2}}$) values found from these graphical plots are given in table 41d. (page 129).


GRAPH 7 Hydrolysis of Sugar Sulphates
1.0
0.8
0.6
0.4
0.2
0
Log a/a-x
1.0
2.0
3.0
4.0
Time (hours)
⊙-glucose-3-sulphate
⊡- compound (S_B)
×- compound (S_A)

Glucose-3-Sulphate		E_{blank} 0.845		E_{24} 0.432		
Time (hours)	0.166	0.333	0.5	1.0	1.5	2.0
E_t	0.740	0.640	0.583	0.499	0.459	0.455
Log $\frac{a}{a-x}$	0.1274	0.2897	0.4370	0.7899	1.185	1.254

table 41a

Compound (S_A)			E_{blank} 0.822		E_{24} 0.414		
Time (hours)	0.25	0.5	1.0	1.5	2.0	3.0	4.0
E_t	0.780	0.750	0.670	0.629	0.591	0.525	0.491
Log $\frac{a}{a-x}$	0.0472	0.0844	0.2025	0.2783	0.3627	0.5654	0.7242

table 41b

Compound (S_B)		E_{blank} 0.845		E_{24} 0.496		
Time (hours)	0.25	0.5	0.75	1.0	1.5	2.0
E_t	0.721	0.672	0.640	0.600	0.585	0.549
Log $\frac{a}{a-x}$	0.1907	0.2974	0.3844	0.5259	0.5935	0.8186

table 41c

Compound	Half-life (hours)
Glucose-3-Sulphate	0.41
(S_A)	1.61
(S_B)	0.80

table 41d

Expt. 44 Examination of Acidic Fraction (S_3)

Fraction (S_3) was a complex mixture (Expt. 41; page 123), which, after successive separations on 3MM filter sheets, gave rise to a small amount (ca. 5 mg.) of an ionophoretically homogeneous substance (S_C). This had M_G 0.80 and gave a pink colour with aniline oxalate.

It had a degree of polymerisation[82] of 2.03, and on acid hydrolysis, it gave rise to arabinose[81] (2 moles) and sulphate.[79] (1 mole). On this basis, that is calculated for the ammonium salt of a monosulphated arabinobiose, it had $[\alpha]_D + 165^\circ$. (c. 0.14 in water).

Methylation[84] of compound (S_C) (1-2 mg.), methanolysis and gas-liquid chromatographic examination[85] gave peaks characteristic of methyl 2,4- and 2,3-di-O-methyl arabinosides.

Desulphation of (S_C) (1-2 mg.) with methanolic hydrogen chloride (3%; 0.5 ml.) overnight at room temperature was followed by methylation, methanolysis and gas-liquid chromatographic examination in the usual way. This showed peaks corresponding to methyl 2,3,4-tri- and 2,3-di-O-methyl arabinosides. It follows therefore, that the sulphate residue is situated at C_3 of the non-reducing portion of the disaccharide, and that the glycosidic linkage is accordingly 1,4- or 1,5-. An attempt was made to distinguish between these two possibilities as follows:-

Compound (S_C) (ca. 1 mg.) was dissolved in water (10 drops) containing bromine (2 drops) and calcium carbonate. (10-20 mg.). This mixture was kept in the dark at room temperature for 72

hours, and then taken to dryness, dissolved in dimethyl-formamide (0.2 ml.) and methylated in the usual way.[84]

The methylated product was de-ionised with Amberlite IR 120(H) ion-exchange resin, concentrated to dryness, and methanolised for gas-liquid chromatographic comparison with 2,3,4- and 2,3,5-tri-O-methyl arabonolactones. Unfortunately, no positive interpretations could be made from the resultant gas-liquid chromatogram, and so the nature of the glycosidic bond in compound (S_C) remained in doubt.

Expt. 45 Examination of the Neutral Fraction (R) from Partial Acid Hydrolysis

By repeated separation of neutral fraction (R) (2.7 g.; Expt. 40; page 121) on filter paper sheets, small amounts of three oligosaccharides were obtained as follows:-

Compound (R_1) (2.3 mg.) had a degree of polymerisation[82] of 2.05, $[\alpha]_D$ +32° (c. 0.23 in water) and gave rise to galactose only on acid hydrolysis. (solvents 1 and 3; developing reagent 3). It did not migrate on ionophoresis in pyridine-acetic acid buffer (page 41), and had the same chromatographic mobility as authentic 6-O-β-D-galactopyranosyl-D-galactose in solvent 1 (R_{gal} 0.34) and solvent 2 (R_{gal} 0.17).

A portion (1 mg.) of (R_1) was methylated[84] and methanolised in the usual way. Gas-liquid chromatographic[85] examination of the product showed peaks corresponding to the methyl glycosides of 2,3,4,6-tetra- and 2,3,4-tri-O-methyl galactose. The pattern of peaks was identical with that produced by authentic 6-O-β-D-galactopyranosyl-D-galactose after the same treatment.

Compound (R_2) (2.3 mg.) had a degree of polymerisation[82] of 1.90, $[\alpha]_D$ +72° (c. 0.23 in water) and gave mainly galactose on acid hydrolysis. (traces of glucose and arabinose were revealed with alkaline silver nitrate reagent). It was a chromatographically pure syrup (silver nitrate), and had the same mobility as authentic 3-O-β-D-galactopyranosyl-D-galactose in solvent 1 (R_{gal} 0.49) and solvent 2 (R_{gal} 0.24).

Furthermore, it gave a red colour with triphenyltetrazolium hydroxide[88] reagent (which does not react with sugars substituted at C_2), and a grey-green colour with aniline-diphenylamine-phosphoric acid reagent.[89] With this latter reagent, authentic 4-O-α-D-galactopyranosyl-D-galactose and other 1,4- linked oligosaccharides, gave bright blue colours, whereas 3-O-β-D-glucopyranosyl-D-glucose (laminaribiose) and authentic 3-O-β-D-galactopyranosyl-D-galactose gave grey-green colours.

Methylation of compound (R_2) (1 mg.) and examination in the usual way gave peaks characteristic of the methyl glycosides of 2,3,4,6-tetra- and 2,4,6-tri-O-methyl galactose.[85] Compound (R_3) (2.7 mg.) was not a pure syrup. It had $[\alpha]_D$ +74° and gave mainly a pink spot (aniline oxalate) with Rgal 0.77 (solvent 1) and 0.73 (solvent 2). It also gave rise to major proportions of arabinose on acid hydrolysis, together with smaller amounts of galactose and glucose. Its chromatographic mobility was distinctly different from authentic 3-O-β-L-arabinopyranosyl-L-arabinose (R_{gal} 0.82 in solvent 1) run as a control.

Expt. 46 Fragmentation of Polysaccharide (A_1) by Partial Acetolysis

Polysaccharide (A_1) (12 g.) was dried for 24 hours, and added in portions to a rapidly stirred solution of acetic anhydride (250 ml.), glacial acetic acid (250 ml.) and concentrated sulphuric acid (25 ml.). The mixture was maintained at 0°C for 3 hours with vigorous stirring, and then agitated on a mechanical shaker for 70 hours at room temperature.

Insoluble substances were removed by centrifugation, and the dark brown supernatant was diluted with ice-cold water (4 litres) and neutralised (pH 4-5) by addition of sodium bicarbonate.

Syrupy sugar acetates were precipitated and partially removed by centrifugation and then dried to a grey amorphous powder. (2.3 g.). The supernatant was concentrated to a small volume, filtered to remove precipitated salts, and then both filtrate and salts were extracted with chloroform. (4 x 500 ml.). The chloroform solution was concentrated, and evaporated under high vacuum to remove last traces of acetic anhydride, leaving an amorphous powder. (7 g.). This residue was combined with the first grey powder (2.3 g.), dried and dissolved in dry methanol (150 ml.) containing dry chloroform. (10 ml.).

A solution of dry barium oxide (1.9 g.), in dry methanol (50 ml.) at 0°C, was filtered and added to the methanolic solution of the sugar acetates. This mixture was set aside

at 0°C for 20 hours.

The de-acetylated mixture, obtained in this way, was neutralised to pH 6 with sulphuric acid, concentrated, taken up in water and filtered. The filtrate was de-ionised with Amberlite IR 120(H) ion-exchange resin, and the acidic solution was shaken with Amberlite LA-2 liquid anion exchanger (5% in chloroform; 4 x 30 ml.) until the aqueous layer was neutral. The neutral solution was washed with chloroform and concentrated to a viscous syrup (T). (3.4 g.).

Chromatographic examination of this syrup (T) indicated that only small quantities of slow oligosaccharides were present, although a new substance (T_1), which gave a pink colour with aniline oxalate, was present in relatively high proportion. Compound (T_1) had a chromatographic mobility (R_{gal} 1.95 in solvent 1) comparable with that expected of such substances as 5-O-L-arabinofuranosyl-L-arabinose and 3-O-L-arabinofuranosyl-L-arabinose.[151] Syrup (T) was therefore examined further.

Similar acetolysis procedures carried out for 24, 48, 60 and 96 hours gave essentially the same results.

Expt. 47 Examination of Syrup (T)

The syrup (3.4 g.) was applied to a charcoal/Celite column (1/1; 25 x 4 cm.) and eluted, in the first instance, with water (1 litre), and then gradientwise with aqueous ethanol. (0.5%-8%; 4 litres). In this way, an oligosaccharide fraction (240 mg.; 7%) was obtained.

This contained a complex mixture of oligosaccharides, two of which, (T_2) and (T_3), gave brown spots (aniline oxalate) on a paper chromatogram. They had chromatographic mobilities comparable with 6-O-β-D-galactopyranosyl-D-galactose and 3-O-β-D-galactopyranosyl-D-galactose respectively, in solvents 1 and 2. (cf. compounds (R_1) and (R_2); pages 131-132).

Compound (T_1) (page 134) was present in the monosaccharide (aqueous) fraction. It had a degree of polymerisation of 1,[82] and remained unchanged on acid hydrolysis.

Methods are presently under consideration to establish the identity of compound (T_1).

Expt. 48 Examination of Compounds (T_2) and (T_3)

The syrup (T) was a considerably more complex mixture than the neutral syrup (R) obtained from aqueous hydrolysis. (Expt 45; page 131). Minute quantities of compounds (T_2) and (T_3) were obtained only after extensive separations on 3MM paper sheets.

Compound (T_2) (ca. 1 mg.) gave rise to galactose and traces of glucose and arabinose on acid hydrolysis. Chromatographic examination in solvent 1 showed that a major substance, (R_{gal} 0.33), identical with 6-O-β-D-galactopyranosyl-D-galactose and compound (R_1) (Expt. 45; page 131), and a minor substance (R_{gal} 0.41), were present.

Compound (T_3) (1.5 mg.) had a degree of polymerisation[82] of 1.98, $[\alpha]_D$ +68° (c. 0.15 in water) and gave rise to galactose on acid hydrolysis. It had chromatographic properties identical

with those of compound (R_2) (page 132), and like that substance, gave colour reactions with triphenyltetrazolium hydroxide[88] and aniline-diphenylamine-phosphoric acid[89] reagents, consistent with a 1,3- linked galactobiose.

Methylation[84] of compound (T_3) (0.5 mg.), methanolysis and gas-liquid chromatographic[85] examination in the usual way, confirmed the presence of 1,3- linked galactobiose, when peaks, characteristic of the methyl glycosides of 2,3,4,6-tetra- and 2,4,6-tri-O-methyl galactose, were observed.

An examination of the remainder of the syrup (T) suggested that it contained arabinose containing oligosaccharides. No evidence for the presence of a true hetero-oligosaccharide was obtained.

Discussion

The genera of green seaweeds, Cladophora and Chaetomorpha, are sometimes considered together as members of the Cladophoraceae, and so, it is of interest to discover whether this close botanical relationship is complemented by similarities in the products of the plants' metabolism.

All samples of dried seaweed were decolourised with organic solvents before examination. Aqueous butanol and acetone were found to be the most effective for Cladophora rupestris and Chaetomorpha linum. These procedures did not remove polysaccharide material. (Expt. 15).

Treatment of the residual, decolourised weeds with hot water gave the best yields of sulphated polysaccharide, very little of this material being extracted with cold water.

Starch-type polymers were present in each of the hot water solutions, since these gave intense blue colours with iodine. The starch-type polysaccharides from Cladophora rupestris and Chaetomorpha capillaris have already been described in Part One of this thesis. During the isolation of the polysaccharides from Chaetomorpha linum, the starch-type polysaccharide was inadvertently degraded, and no material was obtained for a structural investigation. Collection of quantities of pure samples of this particular species of weed is very difficult, and therefore a study of its starch-type polymer was not possible.

The starch-free polysaccharides (A_1) (Cladophora rupestris),

(A_2) (Chaetomorpha capillaris) and (A_3) (Chaetomorpha linum) were heavily contaminated with protein. (ca. 19-25%; table 14; page 90). These values are similar to that found in extracts of Codium fragile[28], and greater than those of Enteromorpha compressa[76] and of Ulva lactuca[77] extracts. (ca. 10%).

Polysaccharides (A_2) and (A_3) were treated with trichloroacetic acid, and this probably accounts for their slightly lower protein contents, since this reagent has previously been found to be effective in de-proteinising other extracts of Cladophora rupestris[73]. It should be remembered that the samples of polysaccharide (A_1) used for purification and fractionation experiments were not treated with trichloroacetic acid. (page 89). Polysaccharides (A_1), (A_2) and (A_3) were similar in other respects as shown in table 14. (page 90). For example, they contained ester sulphate (12.3-15.5%) and had similar specific rotations ($+53^{\circ}$-$+75^{\circ}$) and similar total carbohydrate contents. They also gave rise to the same sugars on acid hydrolysis, although polysaccharide (A_1) contained greater proportions of galactose than polysaccharides (A_2) and (A_3). (table 15; page 93). This feature will be mentioned again later. None of the polysaccharides contained detectable amounts of 3,6-anhydrogalactose, uronic acid or amino sugar.

1. Fractionation Experiments

Previous studies[73] with the sulphated polysaccharide from Cladophora rupestris strongly indicated that the constituent sugars were combined together in one heteropolysaccharide,

but no unequivocal evidence to confirm this was advanced.

Another great obstacle in these studies was the high protein content of the extracts, and although numerous purification methods were tried, none was truly satisfactory. This problem of the heterogeneity of the Cladophora polymer, that is whether it is a true heteropolysaccharide or a mixture of sulphated galactans, arabans and xylans etc., was re-examined in the light of newer fractionation methods, involving ion-exchange materials.

For this work, ion-exchange derivatives of cellulose[153] (Whatman)[154] and Sephadex (Pharmacia) were used. These substances are prepared by the insertion of ion-exchange groupings within the appropriate derivatives of cellulose, on the one hand, and cross-linked dextrans on the other. (Dextrans are basically polymers of α-1,6- linked D-glucose residues). These substances combine ion-exchange properties with a large surface area, and so are suitable for adsorbing polymeric molecules. Sephadexes of different porosities are prepared by varying the degree of cross-linking. In general, materials of low cross-linkage have been found most suitable for chromatography of large molecular weight substances (>10, 000), since in these the gel grains are more accessible to large molecules.

Both cellulose and Sephadex derivatives are obtainable as cationic and anionic exchangers. The following derivatives were available in this laboratory. (page 140).

Material	Functional Group	Type of Exchanger
1. DEAE-cellulose	diethylaminoethyl	weak anionic
2. DEAE-sephadex	diethylaminoethyl	weak anionic
3. CM-cellulose	carboxymethyl	weak cationic
4. SE-sephadex	sulphoethyl	strong cationic

Materials 2,3 and 4 were available in quantities suitable for small-scale experiments only.

Although greatest success with these substances has been achieved in separations of amino acids, nucleotides and proteins, some interesting applications to the carbohydrate field have been reported.

Anion exchangers such as DEAE-[155] and ECTEOLA-[156] (epichlorohydrintriethanolamino) cellulose and DEAE-sephadex[157] have been employed to separate mixtures of acidic polysaccharides, as well as mixtures of neutral and acidic polysaccharides.

Basically, neutral polymers are not usually adsorbed on the ion-exchanger in salt (e.g. chloride) forms, whereas acidic polymers are. Neutral polysaccharides may be adsorbed when special complexing effects (e.g. with borate) are present, or when the exchanger is used in the free base form. Acidic polymers may be recovered by gradient[150] or graded elution with salt solutions of increasing strength, so that the inorganic anions compete with the polymer anions for the sites on the exchanger. Alternatively, application of an alkaline eluting medium will suppress the ability of the exchanger to form salt bonds, and so adsorbed polysaccharides can be recovered in the effluent from the columns.

It should be stressed however, that each particular problem will have its own requirements of concentration and pH of eluting solutions for successful resolution.

By means of these anionic exchange substances, it was considered that a simultaneous fractionation of polysaccharide (A_1) and purification from proteinaceous substances might be achieved.

Before experiments with columns were carried out, efforts were made to find conditions suitable for chromatography of polysaccharide (A_1) on DEAE-cellulose paper, since it was felt that this might provide a useful technique for comparison of later column fractions. (Expt. 19; page 93). Unfortunately, detection difficulties, and problems in distinguishing areas of sulphated polymer led to abandonment of this idea.

Ringertz and Reichard[156] have reported that optimum separation of sulphated mucopolysaccharides occurred with chloride solutions at acidic pH values. Similarly, Schmidt[157] has described experiments, in which separations of these same substances were sharpest on DEAE-sephadex with sodium chloride solutions containing hydrochloric acid. (0.01 molar).

The preliminary experiments were therefore based on the experimental conditions of these workers, using columns DC-I, DC-II and DS-I. (Expt. 20; page 94). Column DC-II was prepared in an alternative salt form (phosphate), buffered at a higher pH, for comparative purposes.

Each column produced essentially the same separation diagrams.(graphs 3, 4 and 5; facing page 96). The emergence

of a readily elutable fraction, virtually free of galactose and arabinose, was an interesting feature. The remainder of the sulphated polysaccharide was eluted as a broader band when the salt concentration was greater than 0.5 molar. These fractions contained the same monosaccharide constituents as the original polysaccharide (A_1). (table 16; facing page 97).

Although an additional quantity of galactose/arabinose rich material could be obtained by stripping the column with sodium hydroxide (0.5 molar), this procedure caused degradation of the exchanger, and so this was omitted in later experiments.

Further efforts were made to acquire larger quantities of galactose/arabinose free material by successive separations on DEAE-sephadex columns DS-II and DS-III. (Expt. 21; page 97). This showed that the technique might be useful, if the effects were reproducible on larger columns of DEAE-cellulose.

A larger column of DEAE-cellulose (DC-III) was therefore prepared in the chloride form. (Expt. 22; page 99). The presence of hydrochloric acid in the eluant was omitted, since this would have led to difficulties in working up large volumes of solution. This omission did not visibly affect the separation, since similar elution patterns (graph 6; facing page 99) were obtained.

The fractions were subdivided to give four separate polysaccharide samples (B_1), (C_1), (D_1) and (E_1), the last three being subdivisions of the main peak. (table 19; page 99). The recovery of material was poorer than expected, although this is a common feature of such columns. A second

fractionation with twice the quantity of polysaccharide (A_1) was made on the same column, after regeneration. The recovery in this case was even poorer, although the separation was similar. Polysaccharide fractions (B_2), (C_2), (D_2) and (E_2), analogous to those obtained in the first separation were isolated. Each set of fractions produced similar proportions of monosaccharides on acid hydrolysis, and so similar polysaccharides were combined.

Polysaccharide (B) constituted a very small proportion of the recovered material and was contaminated with galactose and arabinose. It was decided, at this stage, not to pursue the numerous tedious separations which would be required to produce sufficient quantities of this fraction.

The main fraction, polysaccharide (D) still contained a high proportion of nitrogenous substances (16.9%; table 20; page 100), and so other methods of purifying polysaccharide (A_1) were tried.

Firstly, the combined effect of charcoal (to give an initial decolourisation) and sulphoethyl sephadex was studied. (Expt. 23a; page 101). Sulphoethyl-sephadex (page 140) is a strong cationic exchanger and as such, should be fully ionised at low pH values. Since this material has been reported to possess a high capacity for protein molecules, it was reasoned that any proteinaceous substances in the polysaccharide solution would have a great affinity for the SE-sephadex at low pH values (below the isoelectric point of the proteins), and would therefore be readily separable from the anionic sulphated

polysaccharide, which would have no affinity for the exchanger. In the presence of a large excess of competing cation exchanger, good purification might then be expected.

The recovered polysaccharide (F) obtained when buffers of pH 4.6 were used, was partly purified, since it contained a lower proportion of proteinaceous contaminant (14.4%; table 21; page 101), as compared with polysaccharide (A_1). (25.6%; table 14; page 90). It also contained a higher relative proportion of carbohydrate (49.6%) and ester sulphate (17.0%) than (A_1). (43.3% and 12.3% respectively). However, this material was recovered in very poor yield, 30% after allowance for reduction in protein and ash contents.

Another column of SE-sephadex was prepared to examine its effect alone, and to improve the purification by use at lower pH (3.4) values. (Expt. 23b; page 102). A column of carboxy-methyl-cellulose, buffered at pH 6.0, was examined in a concurrent experiment. (Expt. 23c; page 102).

It was observed that each material adsorbed a substantial proportion of the brown colouring matter from the polysaccharide solutions. The recoveries of polysaccharide were still rather low, and in the former case, the "apparent" protein contamination was increased. This result is rather puzzling, especially since the CM-cellulose treatment was partially successful. (residual protein 19.4%; page 103). The explanation may, in some manner, lie in the different gel-grain physical structure of the sephadex material.

Investigation of the phenol extraction technique[143] was

abandoned because of the formation of intractable emulsions.

To summarise, at this stage, it is evident that the use of ion-exchange derivatives, for fractionation of polysaccharide (A_1), have not fulfilled the promise which they showed in small-scale experiments. It is therefore concluded that their use is not suited, under these experimental conditions, for isolating the preparative quantities of polysaccharide which are required for complete structural investigations. Apart from the appearance of a small amount of material almost free from galactose and arabinose, no major evidence has been obtained that the extract is a mixture of polysaccharides.

The experiments have served to emphasise the tenacity with which the proteinaceous substances are bound up with the polysaccharide material. Whether these are inter-linked by true chemical bonds or by secondary forces, such as salt or hydrogen bonding effects, is still open to question.

The three subfractions (C), (D) and (E) from the main peak (graph 6; facing page 99) of DC-III (Expt. 22; page 99) were examined further. (Expts. 24-28).

As might be expected, they gave rise to similar molar proportions of sugars on acid hydrolysis. (tables 23, 24 and 29). They also showed some gradation in sulphate content, according to the order in which they were eluted from the column. (11.3-14.0%; table 20; page 100). These figures suggest that the polysaccharide may be composed of a spectrum of molecules of comparable, though slightly different, sulphate contents. From this point of view, further chromatographic studies of

polysaccharide (D) might have been interesting, but time did not permit these.

The greater discrepancy in sulphate content between polysaccharide (C) and the others can partly be explained by its higher proportion of glucose (2.5%; Expt. 24; page 103), which is presumably unsulphated. In agreement with the lower sulphate and higher glucose contents, polysaccharide (C) had a higher total carbohydrate content (52.1%) than polysaccharide (D). (49.1%; table 20; page 100).

Treatment of polysaccharide (C) separately with salivary α-amylase and sodium metaperiodate gave residual polymers containing 1.7% and 0.5% of glucose respectively. (Expt. 24; pages 103-104). Thus, of the total glucose (2.5%) in polysaccharide (C), 32% is degraded by α-amylase (i.e. α-1,4- linked as in starch) and 20% resists attack by periodate (e.g. β-1,3- linked as in laminarin). The remaining 48% is unattacked by α-amylase, but is cleaved by periodate, such as would be expected of cellulose. (β-1,4- linked glucan).

These experiments are therefore in keeping with other evidence that Cladophora synthesises a laminarin-type polymer[73] and a cellulosic material,[19] in addition to a starch-type polysaccharide.[3]

It is surprising however, that these glucans (which are almost certainly neutral) have not separated from the strongly acidic polysaccharide. Clearly, the use of DEAE-cellulose as a test of polysaccharide homogeneity must be made with caution.

Polysaccharides (D) and (E) had similar sulphate contents

and gave similar molar proportions of sugars on acid hydrolysis, and it is probable that they differed little in constitution, if at all. It was surprising that they (and polysaccharide (C)) contained a greater proportion of xylose than the original polysaccharide (A_1) as follows:-

	Galactose	Arabinose	Xylose
Polysaccharide (A_1)	3.1	3.2	1.0
Polysaccharide (C)	2.1	2.6	1.0
Polysaccharide (D)	2.4	2.7	1.0
Polysaccharide (E)	2.5	2.8	1.0

These are unexpected observations, since the removal of a small amount of xylose-rich material (table 19; page 99) on DEAE-cellulose should have furnished a polysaccharide richer in galactose and arabinose. For example, purification of the sulphated polysaccharide from Codium fragile, on DEAE-cellulose, provided a purified arabino-galactan by removal of xylose units.[28]

This feature was not discovered until an advanced stage in the investigations had been reached. It certainly seems possible that a portion of galactose/arabinose-rich polymer has been strongly adsorbed on the DEAE-cellulose, and this might also partly account for the low recoveries of polysaccharide fractions. It is worth noting that other instances of irreversible adsorption of polysaccharides (e. g. β- linked xylans) on DEAE-cellulose are known.[158] The above example of the purification of Codium sulphated polymer provides another instance of this irreversible action, since in this case, the

xylan could not be recovered.

It is also worth noting that polysaccharide (F) contained increased proportions of xylose (page 22; page 102) as compared with polysaccharide (A_1). It therefore appears that charcoal, as well as DEAE-cellulose, effects a specific removal of galactose and arabinose units. It is interesting, in this respect, that treatment of polysaccharide (D) with decolourising charcoal (Expt. 25a; page 105), gave a purified polysaccharide (G) with little change in the molar proportions of galactose (2.6), arabinose (2.9) and xylose (1.0) (table 26; page 105), although the treatment brought about a substantial purification from proteinaceous substances. (16.9-8.1%; tables 20 and 25). This purification was also reflected in an increased specific rotation ($+66^{\circ}$-$+80^{\circ}$) and increased sulphate content. (13.3% - 19.1%).

The presence of 3,6-anhydrogalactose is common in red seaweed polysaccharides[40,41], and it has also been found in small quantities in the sulphated polysaccharide from the green seaweed, Codium fragile[28]. In certain cases, these residues can arise by desulphation of galactose-6-sulphate units on treatment with alkali. (fig. 5; facing page 15).

Although an alkaline treatment of polysaccharide (G) (Expt. 26; page 105) caused a substantial loss of ester sulphate (19.1 - 13.8%), a neglible concomitant formation of 3,6-anhydrogalactose (ca. 0.2%) took place. This indicates that very few of the galactose-6-sulphate units (see Expt. 43; page 124) can exist as end-units or as 1,4- linked residues

(such as occur in λ-carrageenan) within the polysaccharide. The confirmation of the presence of an alternative type of alkali-labile sulphate will be discussed later.

Application of the Smith degradation technique[145] to polysaccharides can often provide useful information concerning the nature of the glycosidic linkages, especially those present in small proportion. The normal procedure entails successive oxidation with sodium metaperiodate, reduction with potassium borohydride to a polyalcohol derivative, and then treatment with mineral acid under mild conditions. In this way, only the true acetal linkages are cleaved, and contiguous periodate resistant units (provided these are present) or polysaccharide "cores" can sometimes be isolated by precipitation with ethanol. These degraded polysaccharides can then be examined by conventional techniques, or even subjected to further degradation in the same way.

Total acid hydrolysis of the intermediate polyalcohol material may also give valuable information, since substances such as glycerol (from 1,6- linked hexose or 1,4- linked pentose units), erythritol (from 1,4- linked glucose units) and threitol (from 1,4- linked galactose units) may be present among the products. In addition, the residual supernatant solution (after precipitation of the degraded material) may contain some of these alcoholic fragments still attached to sugar units. These can be derived from periodate susceptible units attached to periodate resistant units in the polymer.

When one degradation was applied to polysaccharide (E) (Expt. 27; page 107), a small amount of a degraded arabinogalactan (polysaccharide (J)) was isolated, after ca. 0.33 moles of periodate had been reduced per anhydrohexose unit.

The expression of periodate reductions relative to anhydrohexose units is useful for comparisons within the same heteropolymers.

Polysaccharide (J) gave rise to slightly greater quantities of galactose (1.2 moles) than arabinose (1.0 mole) (table 31; page 108) on acid hydrolysis, indicating that a greater number of arabinose units than galactose units had been cleaved. The chromatographic examination also showed that practically all of the xylose units had been cleaved. The lower rotation of polysaccharide (J) ($[\alpha]_D$ +38°), as compared with polysaccharide (E) ($[\alpha]_D$ ca. +50°), is in keeping with the cleavage of β-linked L-arabinose units.

An acidic hydrolysate of the polyalcohol, obtained on borohydride reduction, contained two major non-reducing substances, in addition to the other sugars. These non-reducing compounds had chromatographic mobilities (solvent 1) comparable with glycerol, on the one hand, and with erythritol and threitol, on the other. The same substances were present in the supernatant solution which was left after precipitation of the degraded polymer.

As indicated previously, glycerol could have arisen from either 1,6- linked hexose or 1,4- linked pentose units, and further evidence for the presence of each of these units will

be discussed later. Erythritol and threitol could arise from 1,4- linked glucose and 1,4- linked galactose units respectively. However, an unambiguous interpretation cannot be made until these non-reducing substances are identified completely.

The destruction of between one third and one quarter of the polysaccharide in this experiment, suggests that some of the galactose units were also cleaved during the oxidation.

It was thought that a comparison of the methylation products of the degraded polysaccharide (J) and the unoxidised polysaccharide (E), might provide a clue as to the glycosidic linkages of the cleaved residues, but both products were too complex for analysis.

2. Desulphation and Partial Fragmentation Studies

The successful application of methanolic hydrogen chloride as a desulphating agent for the sulphated polymers of Enteromorpha compressa[76] and Ulva lactuca[77] has already been described. (pages 30-33).

Treatment of polysaccharide (A_1) with methanolic hydrogen chloride for 96 hours (Expt. 29; page 109), gave a low yield (ca. 20%) of a residual polysaccharide (K). (SO_3 2.3%). It is interesting to compare the molar proportions of galactose, arabinose and xylose in polysaccharide (K), with those of Fisher's[73] trichloroacetic acid (TCA) treated polymer, and with untreated polysaccharide (A_1). These are shown on the following page.

	Galactose	Arabinose	Xylose
Polysaccharide (A_1)	3.1	3.2	1.0
Polysaccharide (K)	2.9	3.7	1.0
TCA Treated Polymer	2.8	3.7	1.0

These figures indicate that acidic reagents bring about a preferential cleavage of galactose and xylose units from the polysaccharide.[73] Fisher also noticed that residues of galactose and xylose were released first on aqueous hydrolysis. This was confirmed when it was found that the reaction with methanolic hydrogen chloride for 24 hours caused liberation of major quantities of galactose and xylose, and only traces of arabinose. (table 32; facing page 110).

It is interesting, too, that the trichloroacetic acid treated Chaetomorpha polysaccharides (A_2) and (A_3) contain the same proportions of arabinose (3.7 moles) and xylose (1.0 mole) (table 15; page 93), as the acid treated Cladophora samples, but have lower proportions of galactose, 1.4 moles and 1.8 moles respectively.

At this point, it is relevant to consider the infra red spectra of polysaccharides (A_1), (A_2), (A_3) and desulphated (A_1), in the region, 820 cm.$^{-1}$ - 850 cm.$^{-1}$ The specific absorption bands of sulphated esters of glucose and galactose have already been mentioned. (pages 24 and 28). Thus, absorption bands at 820 cm.$^{-1}$, 830 cm.$^{-1}$ and 850 cm.$^{-1}$ are thought to be characteristic of primary sulphated hydroxyl, secondary equatorial sulphated hydroxyl and secondary axial sulphated hydroxyl respectively.[72]

Polysaccharide (A_1) showed a broad absorption band at 810 cm.$^{-1}$ - 840 cm.$^{-1}$, suggesting that both equatorial and primary sulphated hydroxyls were present. On the other hand, the Chaetomorpha polysaccharides (A_2) and (A_3) exhibited a peak absorption at 825 cm.$^{-1}$ - 835 cm.$^{-1}$, suggesting that these polymers contained a smaller proportion of primary sulphated hydroxyl. This is possibly in keeping with the lower galactose contents of these polysaccharides, if an infra red absorption at 820 cm.$^{-1}$ is associated with galactose-6-sulphate residues.

The spectrum of the partially desulphated polysaccharide (K) (Expt. 29; page 110) was distinctly different from that of the parent polysaccharide (A_1) in possessing a sharp and diminished peak at 819 cm.$^{-1}$, and no absorption in the 830 cm.$^{-1}$ region. This is in agreement with the expectation that primary sulphated hydroxyl, such as galactose-6-sulphate, would show greater resistance to acidic reagents, than secondary equatorial sulphated hydroxyls.[51] (see Expt. 43e; page 127). Similar modifications of the infra red absorption bands of carrageenan have been observed by Dolan and Rees,[49] during removal of ester sulphate from that polysaccharide.

These observations were supported, to some extent, by a study of the infra red spectrum of arabinose-3-sulphate itself. (see Expt. 43; page 124). This contained three bands in the region, 750 cm.$^{-1}$ - 860 cm.$^{-1}$, at 770 cm.$^{-1}$, at 852 cm.$^{-1}$ - 855 cm.$^{-1}$ and a broader band at 818 cm.$^{-1}$ - 830 cm.$^{-1}$

The main band (818 cm.$^{-1}$ - 830 cm.$^{-1}$) may be tentatively

correlated with an equatorially disposed sulphate grouping (cf. glucose-3-sulphate), although it is shifted slightly to longer wavelengths. This is in keeping with Reeves'[159] suggestion that the most stable conformation of the L-arabinopyranose unit is the Cl, in which the C_3 hydroxyl has an equatorial disposition. This feature, concerning the preferred conformation of the arabinose unit, will be mentioned again later, in connection with sodium methoxide treatment of polysaccharide (A_1) (Expt. 35; page 115), and concomitant formation of 2-O-methyl-L-xylose residues. The significance of the small peak at 770 $cm.^{-1}$ is not known, but the third peak, at 852 $cm.^{-1}$ -855 $cm.^{-1}$, may arise from axially disposed sulphate groupings.

The presence of more than one sulphate absorption band in the spectrum can be explained if different conformations of the sugar are present in its equilibrium mixture. This, of course, would also include furanose ring forms.

It must be emphasised, however, that insufficient work has been carried out on sulphated derivatives of sugars, other than glucose and galactose, to say whether the same empirical rules are generally applicable.

Nevertheless, it is tempting to correlate the polysaccharide bands about 830 $cm.^{-1}$ with arabinose-3-sulphate groupings, and to account for the absence of the minor peaks in the polymer spectrum, it might be argued that the sulphated arabinose units are held in a single conformation within the polysaccharide. Consequently, the infra red absorptions of the monosaccharide

equilibrium mixture, and the sulphated monomer units within the polymer might not be exactly the same.

Oxidation of polysaccharide (K) (Expt. 30; page 111) resulted in the reduction of nearly 0.5 moles of periodate per anhydrohexose unit. The residual oxidised polymer still contained unattacked residues of galactose (1.9 moles) and arabinose (1 mole), indicating that some of both these units may be involved in 1,3- linkages, although it is still possible that some of the arabinose units may be sulphated at C_2 or C_3. The lower proportions of arabinose in the oxidised polysaccharide (K), as compared with the ratio of galactose and arabinose (1 : 1) in the original polysaccharide (A_1), and polysaccharide (J) from the polyalcohol (1.2 : 1.0; Expt. 27; page 108), suggests that periodate susceptible arabinose units have been produced during the desulphation procedure. This is in keeping with the presence of 1,4- or 1,5- linked 2- or 3- sulphated arabinose units in polysaccharide (A_1). Treatment of polysaccharide (G) with alkali brought about a concomitant loss of ester sulphate (5.3%) and decrease in the proportions of arabinose (cf. tables 25-28; pages 106, 107), while treatment of polysaccharide (A_1), (A_2) and (A_3) with sodium methoxide resulted in the formation of monomethyl pentose units within the polysaccharides. (Expt. 34; page 114). These results are also in keeping with the presence of arabinose residues containing ester sulphate in a trans position to a contiguous free hydroxyl grouping, that is, on C_2 or C_3. (see page 14).

Final confirmatory evidence concerning these results

will be discussed later.

The formation of the methyl sulphate esters of polysaccharide (A_1) was studied according to the method of Coleman and his coworkers.[147] (Expt. 31; page 112). Esterification was apparently incomplete, since a totally neutral polymer was not obtained. The doubly esterified sulphate groupings were relatively labile, either to reductive fission (Expt. 32a; page 113) or acidic reagents (Expt. 32 b), but desulphation was accompanied by extensive degradation in each case.

Partial hydrolysis studies on polysaccharides (A_1), (A_2) and (A_3) (Expt. 39; page 119) indicated that it might be possible to isolate monosaccharide sulphates, under suitable conditions. The preliminary investigations suggested that the hydrolysates of each polysaccharide probably contained galactose-6-sulphate and an arabinose sulphated derivative, as well as other sulphated oligosaccharide fragments. (table 37; page 120). Accordingly, a large scale hydrolysis of polysaccharide (A_1) (Expt. 40; page 121) was carried out, and the syrupy mixture of sugars was separated on anion-exchange resin to give an acidic fraction (S) and a neutral fraction (R).

The acidic fraction (S), after further purification on charcoal and filter sheets, yielded three compounds (S_A), (S_B) and (S_C), as their ammonium salts.

Fraction (S_A) was a monosaccharide derivative. It had a specific rotation ($[\alpha]_D$ +51°; cf. D-galactose-6-ammonium sulphate,[48] $[\alpha]_D$ +51°), chromatographic mobility (R_{gal} 0.55 in

solvent 1) and ionophoretic mobility (M_G 1.00), identical with those of authentic D-galactose-6-sulphate. It gave rise to nearly molar proportions of galactose (1.15 moles) and sulphate (1.00 mole), on acidic hydrolysis.

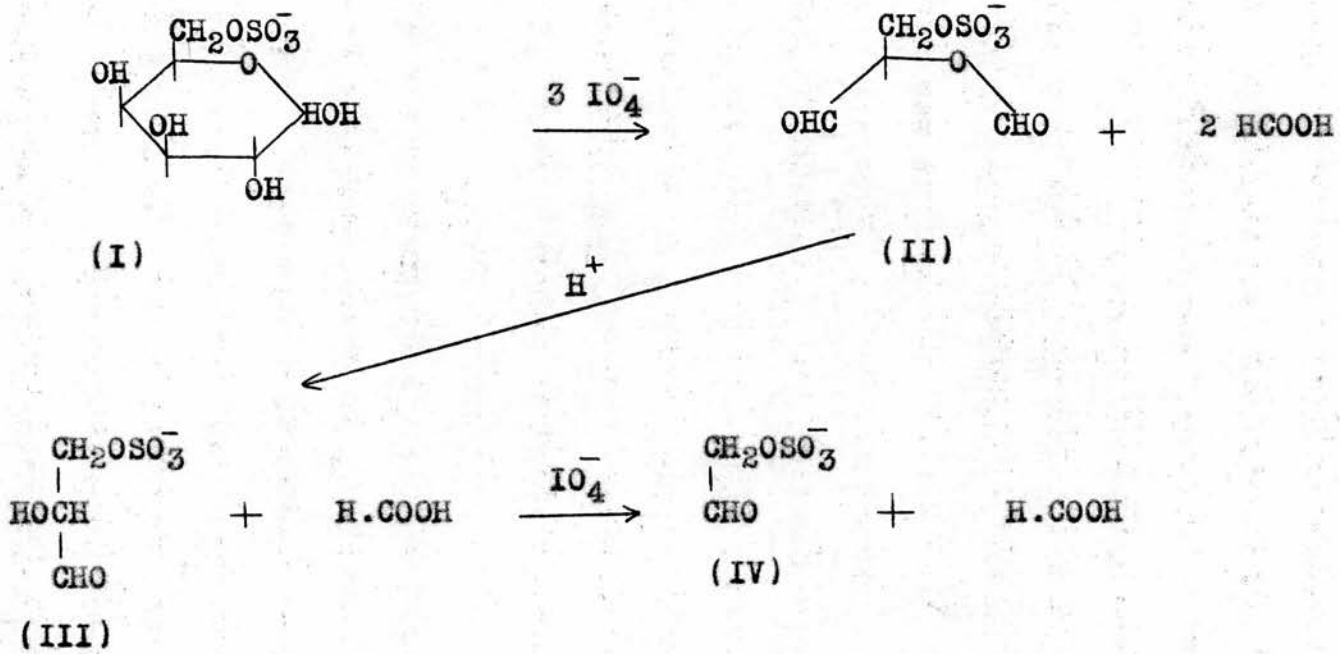
Fraction (S_B), on the other hand, had $[\alpha]_D$ +75°, and gave a pink colour with aniline oxalate. It had M_G 1.18, R_{gal} 0.83 (solvent 1) and gave rise to equivalent proportions of arabinose (1.09 moles) and sulphate (1.00 mole) on acidic hydrolysis. The positive specific rotation suggests that (S_B) is a derivative of L-arabinose.

The following studies (Expt. 43; pages 124-129) confirmed that compounds (S_A) and (S_B) were respectively galactose-6-ammonium sulphate and arabinose-3-ammonium sulphate.

Turvey, Clancy and Williams[148] have shown that, in unbuffered solution, authentic galactose-6-sulphate rapidly reduces 3 moles of periodate (1 hour), and that the reduction of oxidant subsequently becomes steady at 3.5-3.8 moles per mole of galactose-6-sulphate. This behaviour has been verified for L-galactose-6-sulphate, isolated from a partial hydrolysate of the red seaweed polysaccharide, porphyran.[69]

Oxidation of compound (S_A) proceeded in a similar manner, the reduction of periodate becoming steady at 3.7-3.8 moles. (table 39; page 125). The failure to detect any quantities of formaldehyde(tables 40a and 40b; pages 126-127) in the oxidation mixtures, gave further support to the presence of the sulphate grouping on C_6.

The original workers have interpreted this reaction in


CH2OSO3−
OH
O
OH
HOH
OH
(I)
3 IO4−
CH2OSO3−
O
OHC
CHO
+
2 HCOOH
(II)
H+
CH2OSO3−
HOCH
CHO
(III)
+
H.COOH
IO4−
CH2OSO3−
CHO
(IV)
+
H.COOH

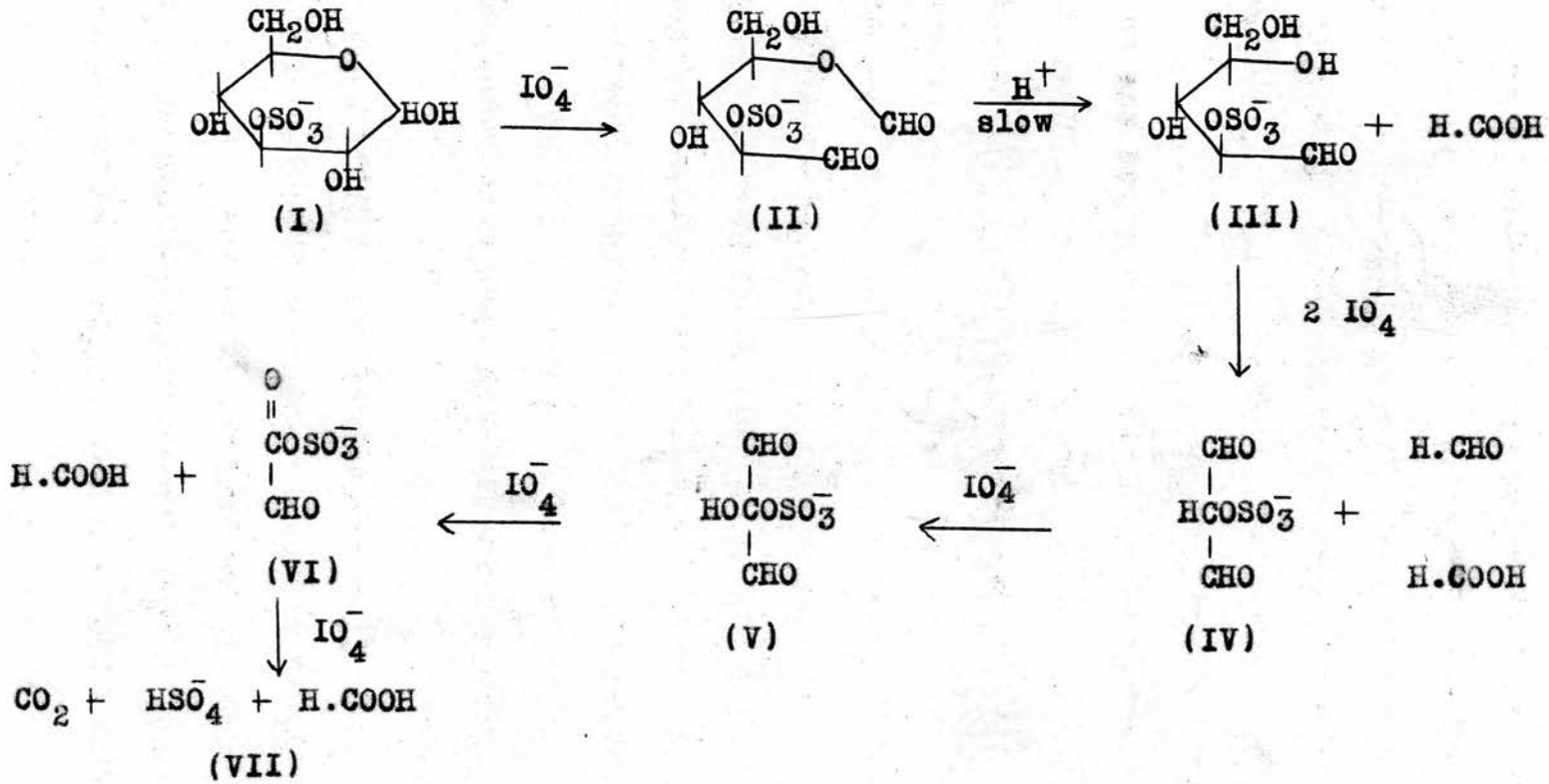
Scheme 1

terms of the accompanying scheme 1.

After the initial oxidation of galactose-6-sulphate (I), hydrolysis of (II) proceeds slowly to give (III), which should theoretically reduce one further mole of periodate, during its conversion to (IV). However, the theoretical reduction of 4 moles of periodate was never observed.

Since no arabinose sulphated derivatives have been studied, as far as the author is aware, periodate oxidation evidence is less diagnostic in this case. However, it was found that, after an initial reduction of nearly 1 mole of periodate (2 hours), oxidation of compound (S_B) proceeded slowly, until, at 192 hours, 3.3 moles of periodate had been reduced. (table 39; page 125). These observations favour a 2- or 3- sulphated arabinose, rather than 4- or 5- substituted, since the last two substances might be expected to reduce 2 moles of periodate fairly rapidly.

Further support for either a 2-sulphate or a 3-sulphate was obtained from the detection of formaldehyde (0.48 moles) in the reaction mixture. (table 40a; page 126). This could not have arisen from an arabinose derivative substituted at C_4 or C_5. In a parallel experiment, glucose-3-sulphate released a similar quantity of formaldehyde (0.38 mole). The yield of formaldehyde, in both cases, is extremely low (theory expects 1 mole), and this suggests that formaldehyde was being consumed in competing side reactions.[160] It is also significant that when galactose-4-sulphate was oxidised, under comparable conditions, only 0.3 moles of formaldehyde


CH2OH
O
OH
OSO3−
HOH
OH
IO4−
CHO
H+
slow
+ H.COOH
(I)
(II)
(III)
2 IO4−
H.CHO
HCOSO3−
(IV)
(V)
(VI)
(VII)
H.COOH +
CO2 + HSO4− + H.COOH

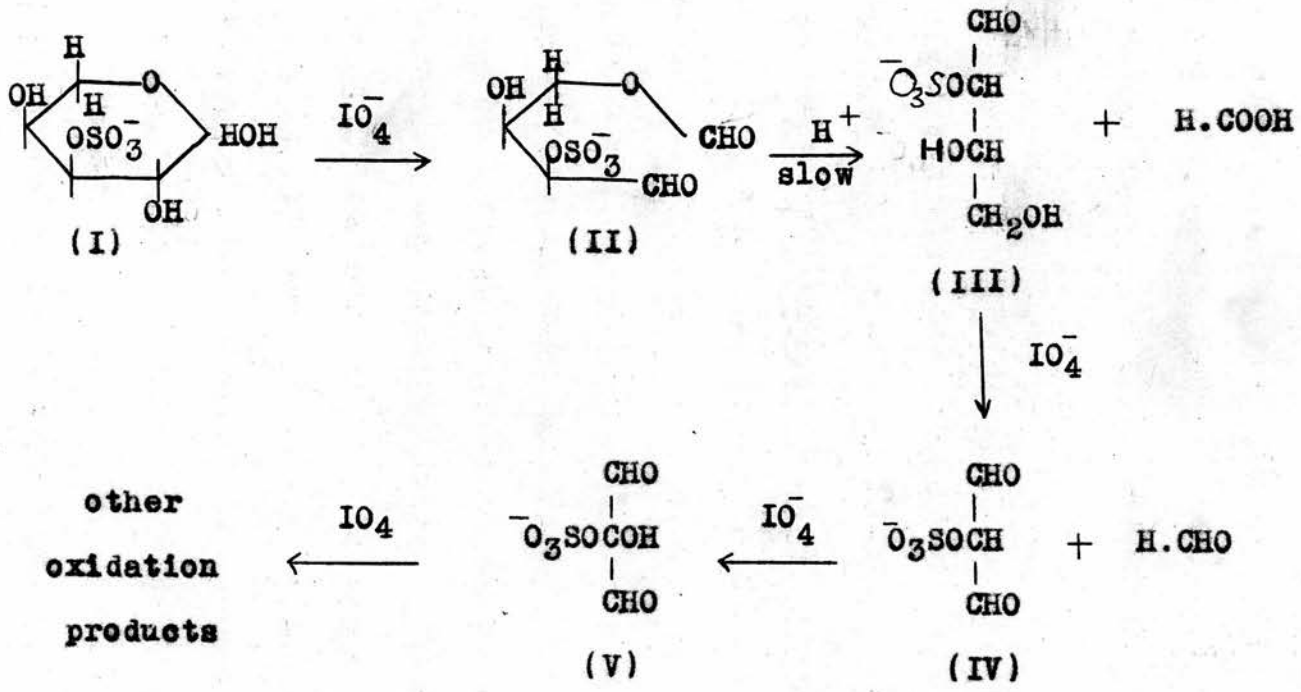
Scheme 2

could be detected.[28] It was found that more quantitative yields of formaldehyde were obtained by oxidising compound (S_B) and glucose-3-sulphate (0.79 moles and 0.81 moles respectively) in solutions, buffered at pH 7.5. (table 40b; page 127). These conditions are known to favour rapid hydrolysis of the formyl ester linkage,[117] which must take place, before formaldehyde can be produced. (steps (II)-(III); scheme 2 and 3).

Turvey and his coworkers have also studied the oxidation[148] of glucose-3-sulphate in unbuffered solution. They found that 1 mole of oxidant was reduced quickly, and thereafter, the reaction proceeded slowly until over 4 moles of periodate had been reduced. These workers have proposed the series of reactions shown in scheme 2, for the approximate course of the oxidation. Hydrolysis of the formyl bond in (II) would give rise to (III), and this would then undergo the series of overoxidation reactions, (III)-(VII), in which 1 mole of formaldehyde would be released, in step (III)-(IV). Unfortunately, these workers did not determine formaldehyde in their experiments.

It should perhaps be emphasised that Turvey regarded scheme 2 as an approximation, and stated that the oxidation did not necessarily follow that exact course.

The oxidation of arabinose-3-sulphate (scheme 3; facing page 160), in unbuffered solution, might then be expected to follow a course similar to that postulated for glucose-3-sulphate, with the production of similar intermediates. Furthermore,



Scheme 3

as was indeed the case, the oxidation might be expected to cease when between 3 and 4 moles of periodate were reduced. (cf. 4-5 for glucose-3-sulphate).

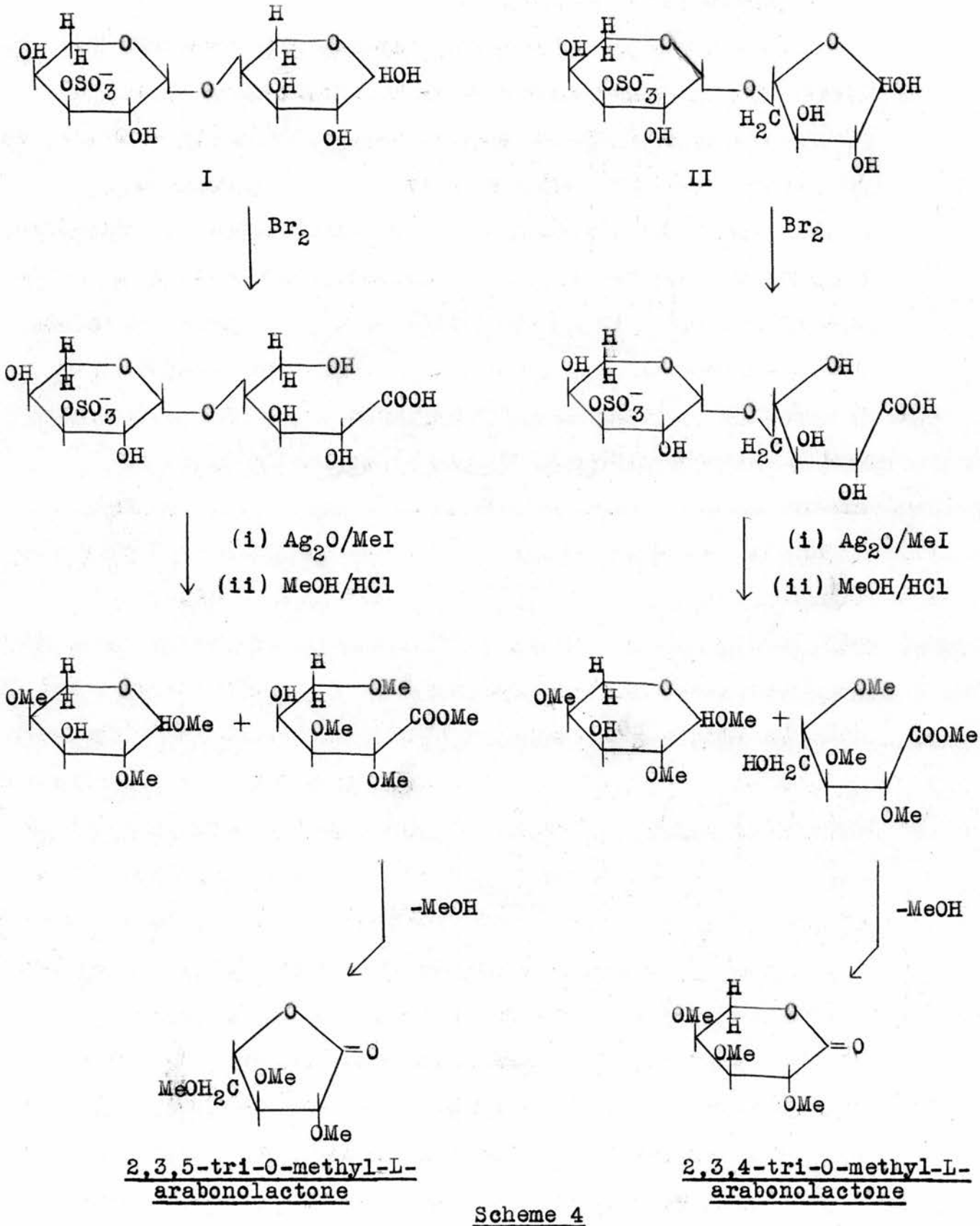
Methylation of compounds (S_A) and (S_B) confirmed their structures as galactose-6-sulphate and arabinose-3-sulphate respectively. Gas-liquid chromatographic examination of the methylated, methanolised substances showed peaks which were characteristic of methyl 2,3,4-tri-O-methyl galactosides (S_A), and methyl 2,4- and 2,5- di-O-methyl arabinosides (S_B).

It has been shown by Rees[51] that the rate of hydrolysis of ester sulphate groupings, within pyranose rings, can be correlated with their disposition in the ring. Thus, primary sulphated hydroxyls are more stable than axial sulphates, which are in turn more stable than equatorial ester sulphates. This is in keeping with the generalities proposed by Barton and Cookson,[161] that equatorially disposed esters, in cyclohexane systems, are more readily hydrolysed than axial ester groupings. In addition, it was shown that, by application of first order rate kinetics, estimates of the half-lives ($t_{\frac{1}{2}}$) of the particular ester sulphates can be made.[51] The values were found to lie in the predicted order of stability, that is, primary hydroxyl > axial > equatorial. (> more stable than).

In the present studies, the value of $t_{\frac{1}{2}}$ found for glucose-3-sulphate was 0.41 hours, for compound (S_A), it was 1.61 hours, and for compound (S_B), it was 0.80 hours. The values for (S_A) and glucose-3-sulphate are close to those quoted by Rees for galactose-6-sulphate (1.55 hours) and glucose-3-sulphate (0.42 hours).

The $t_{\frac{1}{2}}$ value of 0.80 hours, found for arabinose-3-sulphate (compound (S_B)), lies between the figures obtained by Rees for equatorial (0.42 hours) and axial (1.45 hours) sulphates. If arabinose-3-sulphate exists in the pyranose ring form, then the large sulphate grouping would be expected to possess an equatorial disposition, so that a value as high as 0.80 hours is a little unexpected. However, no studies have been carried out with authentic arabinose sulphates, and so no firm predictions can be made concerning their properties. For example, it has already been suggested (page 154) that more than one conformation of arabinose-3-sulphate may exist in its equilibrium mixture, and that this might explain differences between the infra red spectra of the monosaccharide and the polysaccharide. In the same way, the presence of several conformations in the mixture would produce an overall hydrolysis rate, made up of contributions from each conformation, and this might be the reason for the high $t_{\frac{1}{2}}$ value observed.

It has also been shown by Rees, that a study of the sulphate release curves of sulphated polysaccharides may provide a useful indication of the types of ester sulphate which are present in the polysaccharide. For example, the sulphated polymers of Enteromorpha compressa[76] and Ulva lactuca[77] each gave two phase curves, consistent with their containing labile ester sulphate (xylose-2-sulphate) and more stable axial sulphate. (rhamnose-2-sulphate). In contrast, it was found that smooth sulphate release curves could not be obtained on hydrolysis of polysaccharide (A_1) (by Dr. D. A. Rees), suggesting that some


I
II
Br_2
Br_2
(i) Ag_2O/MeI
(ii) MeOH/HCl
(i) Ag_2O/MeI
(ii) MeOH/HCl
-MeOH
-MeOH
2,3,5-tri-O-methyl-L-arabonolactone
2,3,4-tri-O-methyl-L-arabonolactone

Scheme 4

anomalous feature was present.

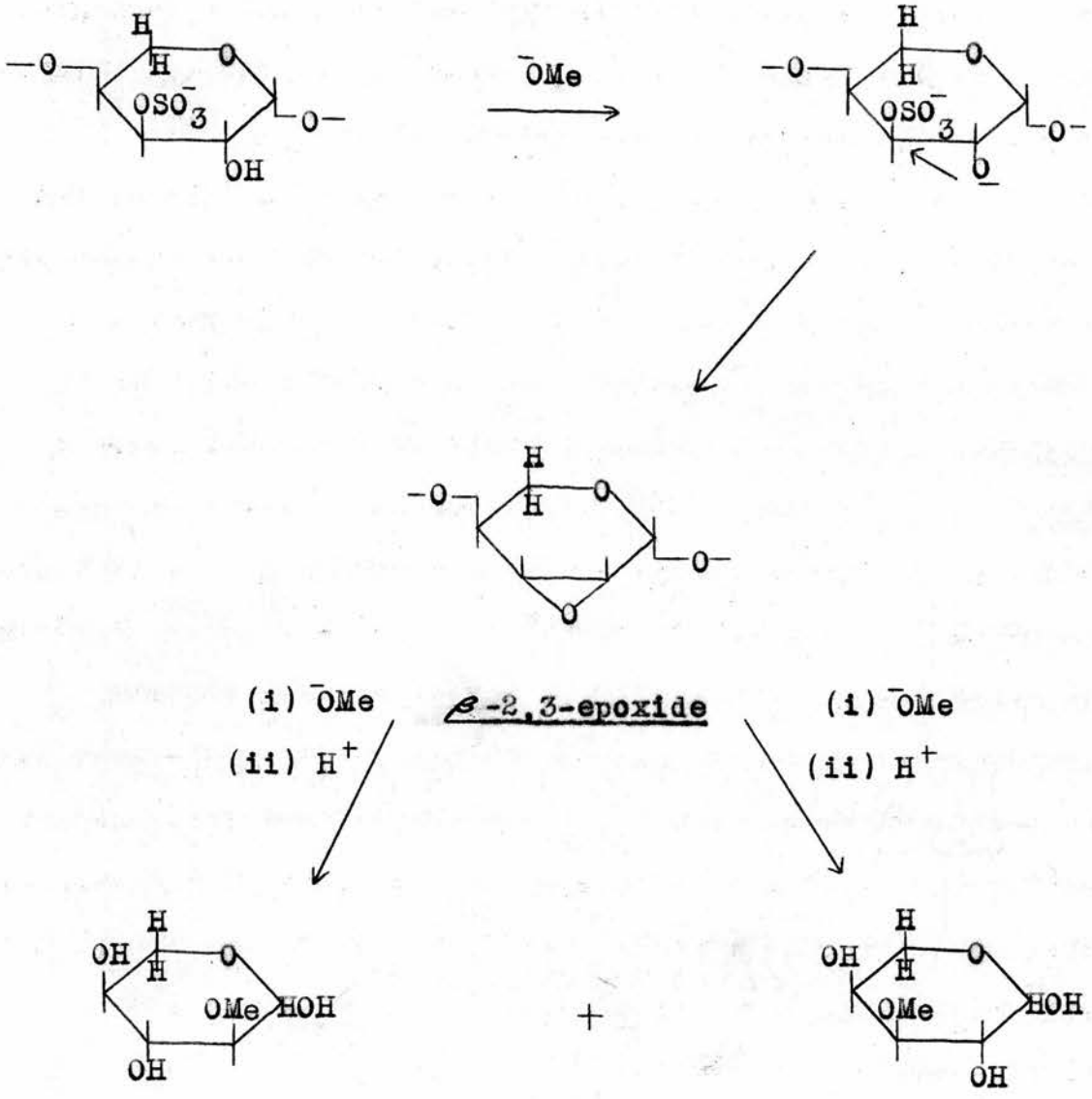
The third acidic fragment, compound (S_C) from fraction (S_3) (Expt. 44; page 130) proved to be an interesting substance. It was a disaccharide which gave rise to arabinose (2 moles) and sulphate (1 mole) on acid hydrolysis. Methylation and methanolysis gave methyl 2,4- and 2,3-di-O-methyl arabinosides, suggesting that the sulphate residue was situated on C_3 of the non-reducing portion of the molecule. The absence of methyl 2,5-di-O-methyl arabinosides (whose formation appears to be favoured in this particular methylation) in this mixture also indicated that this non-reducing moiety had a pyranose conformation. This received support when a desulphated, methylated and methanolised portion of compound (S_C) gave rise to only methyl 2,3,4-tri-O-methyl and 2,3-di-O-methyl arabinosides. On the basis of this evidence, it is tentatively suggested that compound (S_C) has one of the structures I and II, shown in scheme 4, in which the glycosidic linkage is depicted as β-1,4- or β-1,5-. The β-configuration of the glycosidic linkage is suggested because of the high positive rotation of (S_C) ($[\alpha]_D$ +165°), since this is of the order expected for a monosulphated β-linked L-arabinobiose. An attempt was made to distinguish between the two structures I and II by the series of reactions shown in scheme 4. (Expt. 44; page 130).

The products of the reaction with compound (S_C) were subsequently compared, on a gas-liquid chromatogram, with authentic samples of the required isomeric tri-O-methyl arabonolactones. Unfortunately, insufficient product was

formed to give an unambiguous picture, so that the nature of the glycosidic linkage must remain unsettled in the meantime.

It is interesting to compare this type of sulphated oligosaccharide with aldobiouronic acid fragments, in which the glycosidic linkage is stabilised by inductive effects of the carboxyl residue in the non-reducing unit.[162] Foster and Overend[163] and Edward[164] have discussed the stabilising effect of large substituents, such as sulphate, in terms of the effect of their non-bonded interactions on the ability of the species to attain the planar carbonium ion structure, which is postulated as a hydrolytic intermediate. Turvey and his coworkers[50] have shown that the presence of sulphate on C_6 exerts a greater stabilising effect than one on C_3.

The presence of L-arabinose-3-sulphate residues in polysaccharide (A_1) was confirmed by treatment of the polymer with sodium methoxide, on a large scale. (Expt. 35; page 115). Hydrolysis of the resultant polysaccharide (L) and separation of the derived syrup gave a crystalline substance which was identified as 2-O-methyl-L-xylose, since it had the same physical properties as authentic 2-O-methyl-D-xylose, but a specfic rotation opposite in sign. Traces of 3-O-methyl arabinose and 3-O-methyl xylose were also probably formed by this treatment. The same sugars had previously been recognised qualitatively from similar preliminary treatments of polysaccharides (A_1), (A_2) and (A_3). (Expt. 34; page 114). These observations provide another point of similarity between



α-2,3-epoxide

2-O-methyl-L-xylose

3-O-methyl-L-arabinose

Scheme 5

the Cladophora and Chaetomorpha polysaccharides. The formation of these monomethyl pentose derivatives can be readily explained if the reactions take place by means of intermediate epoxide derivatives, as postulated by Peat[165] and Percival.[35] Mechanisms of sugar epoxide formation and subsequent ring opening reactions have been the subject of two recent review articles.[166,167]

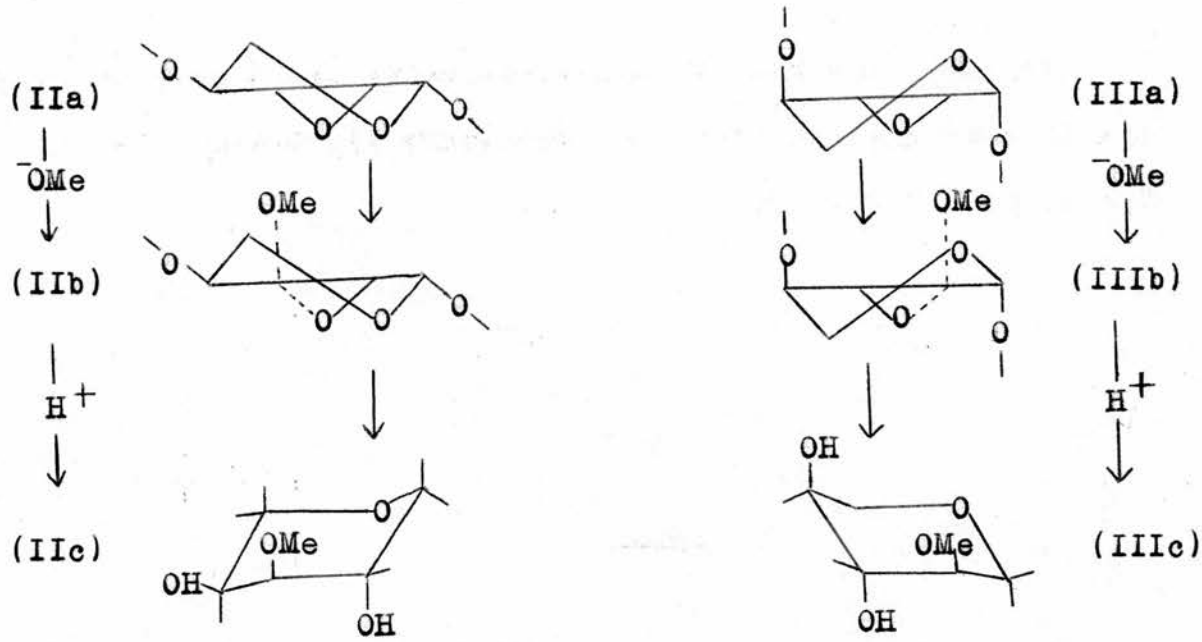
It is generally agreed that the transition states for bimolecular (S_N2) displacement reactions, such as are thought to occur in the formation of most epoxide rings within cyclohexane systems, require a specific trans and diaxial arrangement of four coplanar centres.[166,167] Similarly, when an epoxide ring is opened with nucleophilic reagents, attack will normally take place at the carbon atom which will give trans and diaxial products.[168] (Fürst and Plattner's rule). However, the major product isolated will depend on many factors, including the nature of the transition state,[167] and steric and electronic effects, as well as the predominant conformation of the epoxide.[166] In most ring opening reactions of 2,3-epoxides, attack appears to predominate at C_3,[167] although exceptions have been reported in the case of some 2,3-anhydrofuranose derivatives.

In the present study, the major product, 2-O-methyl-L-xylose, must have arisen by attack of methoxide ion (OMe^-) at C_2. It is interesting that Percival and Wold observed a similar phenomenon when residues of 2-O-methyl-D-xylose were formed within the sulphated polysaccharide from Ulva lactuca.[77] The probable course of the reaction is depicted in scheme 5, for a

β-1,4- linked L-arabinopyranose unit. Although trace quantities of 3-O-methyl arabinose were identified chromatographically, there was not enough of this substance to determine its configuration unequivocally, and it therefore not certain that this is the other expected product. (see scheme 5; facing page 164). However, in view of Percival and Wold's experience, it is considered less likely that 3-O-methyl arabinose arose from 2-sulphated xylose units. The origin of the second trace product, tentatively identified as 3-O-methyl xylose, is also obscure. Presumably, this compound could have arisen from a small amount of 3-sulphated xylose or 2-sulphated arabinose units.

Obviously, reactions in polysaccharide systems lend themselves less to precise mechanistic interpretations, than do their counterparts in the monosaccharide field. For example, it is probable that steric effects are very important in influencing reaction pathways, and there is no way, at present, of gauging the magnitude of these in polysaccharide systems. There is the additional complicating factor, in the present instance, that the arabinose units may exist in either furanose or pyranose forms, or indeed in both.

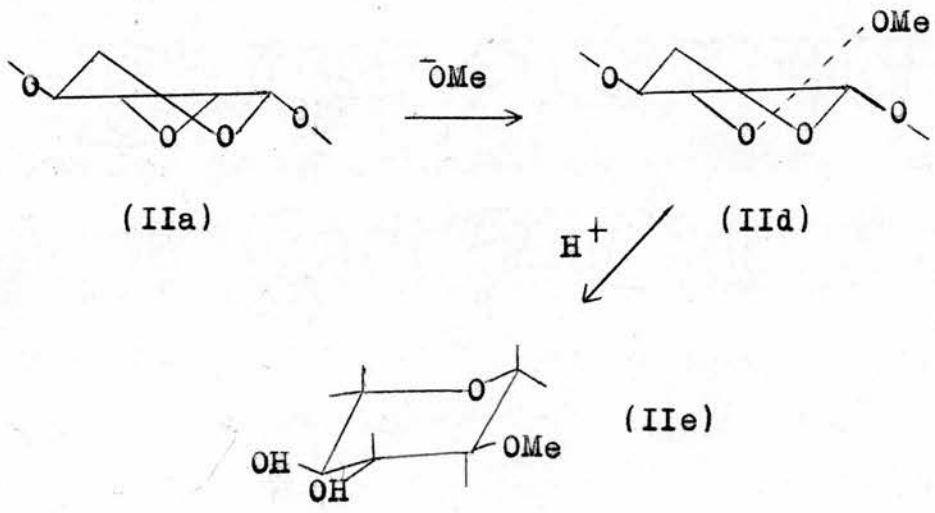
However, insofar as there is evidence that at least some of the 3-sulphated arabinose units exist in the pyranose form (compound (SC); see page 162), it is interesting, though admittedly speculative, to attempt a consideration of the requirements which are needed for the conversion of 3-sulphated L-arabinose units into 2-O-methyl-L-xylose.



3-O-methyl-L-arabinose

2-O-methyl-L-xylose

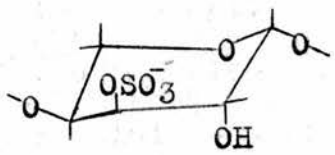
Scheme 6



2-O-methyl-L-xylose

Scheme 7

Firstly, the L-arabinopyranose units would need to adopt the 1C conformation[159] (fig. 15; structure I), favourable for 2,3-anhydro formation.[166]



Structure I

fig. 15

This is probably not the preferred conformation, since there are more axial substituents, including the bulky sulphate grouping, than equatorial.[161] It might also be expected that such an axially disposed sulphate residue would show an absorption at 850 cm.$^{-1}$ in the infra red[72] (see page 154), although this is by no means proved for arabinose derivatives.

On epoxide formation, structure I would give rise to the half-chair form IIa (scheme 6; facing page), which might equilibrate with the alternative half-chair IIIa. There is no means of telling which form would be preferred,[166] but only structure IIIa can undergo attack at C_2 to give a diaxial product with the required L-xylose configuration. (structure IIIc; scheme 6).

However, cases are known where the presence of electronic or steric effects can increase the energy of the pseudo-diaxial transition state (structure IIb; scheme 6) until it is greater than that of the pseudo-diequatorial transition state

Sugar	Location of Sulphate	Source
Galactose	C_2	λ-Carrageenan.[48]
	C_4	Carrageenan and other red algae.[48] The green alga, Codium fragile.[28]
	C_6	Most red algae.[48] Green algae including, Codium,[28] Cladophora and probably Chaetomorpha species.
Rhamnose/Xylose	C_2	Green algae, e.g. Ulva,[77] Enteromorpha[76] and probably Acrosiphonia[75] species.
Fucose	C_4 and possibly C_2 and C_3	Brown algae, especially Fucus[37] species.
Arabinose	C_3	Green algae, e.g. Cladophora and Chaetomorpha species.

table 42

(structure IId; scheme 7; facing page 166), and then di-
[167]
equatorial opening can occur. If such effects are operative here, there might be no need to postulate conversion of structure IIa into structure IIIa (scheme 6; facing page 166), since diequatorial opening of structure IIa, by attack at C_2, would give the required product IIe (scheme 7) in the most
[159]
stable 1C conformation of 2-O-methyl-L-xylose.

Although there is perhaps no justification for suggesting a unique mechanism for this transformation, the principal conclusion of the experiment seems to be unquestionably correct; namely that only 3-sulphated residues of L-arabinose could readily be converted into 2-O-methyl-L-xylose units, under the experimental conditions.

As far as the author is aware, this is the first reported occurrence of sulphated arabinose in natural products, and this emphasises the variety with which monosaccharide sulphates are found in algal structural materials. (see table 42; facing page 167).

A summary of this part of the work, concerning the location of ester sulphate in Cladophora rupestris sulphated polysaccharide, has recently been communicated to a meeting of the Biochemical Society. A reprint of this can be found inside the back cover of this thesis.

The neutral syrup (R) from partial acid hydrolysis was examined primarily for hetero-oligosaccharide fragments (Expt. 45; page 131), since these would provide positive

proof that polysaccharide (A_1) is a true heteropolymer.

Two compounds, (R_1) and (R_2), were identified respectively as 6-O-β-D-galactopyranosyl-D-galactose and 3-O-β-D-galactopyranosyl-D-galactose, on the basis of chromatographic mobility, specific rotation and the products formed on acid hydrolysis and methylation. A third compound (R_3) was probably mainly an oligosaccharide which gave rise to arabinose on acid hydrolysis. It had a chromatographic mobility different from authentic 3-O-β-L-arabinopyranosyl-L-arabinose, which has been isolated from a partial acid hydrolysate of Codium fragile sulphated polysaccharide.[28]

No evidence for the presence of hetero-oligosaccharides was obtained.

The release of oligosaccharides during partial acetolysis of polysaccharide (A_1) was therefore studied. This procedure generally results in liberation of increased quantities of oligosaccharides. Acetylation of low molecular weight fragments renders these more resistant to further degradation, so that products normally susceptible to acidic hydrolysis may then survive. For example, acetolysis, together with simultaneous desulphation, was successfully applied to λ-carrageenan to give good yields of 3-O-α-D-galactopyranosyl-D-galactose.[47]

The main product from polysaccharide (A_1), apart from monosaccharides, was a substance (T_1) (Expt. 46; page 133), which from its chromatographic mobility was first thought to be an arabinobiose, containing furanose ring forms.[151] Subsequent

experiments indicated that (T_1) was not such a substance, since it had a degree of polymerisation of 1 and was unaffected by mineral acid. Experiments are presently being carried out to identify this compound (T_1).

The neutral oligosaccharide fraction was obtained in somewhat poorer yield than expected (7%), and it was an extremely complex mixture. Small quantities of two galactobioses, (T_2) and (T_3) were obtained (Expt. 48; page 135), only after exhaustive separations on filter sheets.

Fraction (T_2) contained two components, but gave galactose on acid hydrolysis. The main substance had a chromatographic mobility (R_{gal} 0.33 in solvent 1) identical with compound (R_1) (Expt. 45; page 131) and with 6-O-β-D-galactopyranosyl-D-galactose. The contaminating minor substance was only apparent with alkaline silver nitrate reagent. It had a chromatographic mobility (R_{gal} 0.41 in solvent 1) different from compound (T_3) and from 3-O-β-D-galactopyranosyl-D-galactose.

Compound (T_3) was probably identical with the compound (R_2) (Expt. 45; page 132) obtained on partial acid hydrolysis. Thus it had a chromatographic mobility (R_{gal} 0.49 in solvent 1) identical with the compound (R_2) and gave rise to only galactose on acid hydrolysis. Its specific rotation ($[\alpha]_D$ +68°) was also comparable with that of compound (R_2) ($[\alpha]_D$ +72°) and with that of 3-O-β-D-galactopyranosyl-D-galactose ($[\alpha]_D$ +63°)[170]. Methylation and gas-liquid chromatographic examination of the products confirmed the 1,3- linkage, when peaks, characteristic of the methyl glycosides of 2,3,4,6-tetra- and 2,4,6-tri-O-methyl

galactosides were observed.

Although several other impure fractions from the oligosaccharide syrup were studied, none of these provided a single pure hetero-oligosaccharide.

The production of ostensibly the same two galactobioses from both aqueous hydrolysates and partial acetolysates lends some weight to the supposition that these are authentic structural features and not acid reversion artefacts, although this possibility cannot be completely ignored.

The presence of β-1,3- linked galactose-6-sulphate units is in keeping with a certain amount of periodate resistant galactose (Expt. 30; page 111), and also with the failure to obtain 3,6-anhydrogalactose on treatment with alkali. (Expt. 26; page 105). It is also in keeping with the identification, by Fisher[73], of 2,4-di-O-methyl galactose among the fragments of the hydrolysed methylated polysaccharide. It is interesting that, as in the sulphated polysaccharide from Codium fragile[28], these galactose residues are probably joined by β-glycosidic bonds. This is in contrast to λ-carrageenan which contains α-1,3-linked galactose units.

The identification of β-1,6- linked galactobiose is in some measure supported by the earlier work, since Fisher and Percival showed that a considerable proportion of the galactose units were cleaved by periodate. In addition, 2,3,5-tri-O-methyl galactose was tentatively identified in the acid hydrolysate of the methylated polysaccharide.

The presence of 3-sulphated arabinose units is also in

agreement with the earlier results. As end groups, these, on methylation, would yield the 2,4-di-O-methyl sugar, and if 1,4- or 1,5- linked, the 2-O-methyl arabinose isolated by Fisher and Percival.[73]

Conclusion

The major component of the water-soluble polysaccharides of Cladophora, after removal of a starch-type polysaccharide, is a complex mixture of galactose, arabinose and xylose, with smaller amounts of glucose and rhamnose. Similar mixtures are obtained from Chaetomorpha, Codium and Caulerpa.

Fisher and Percival, in their original investigations of Cladophora, obtained methylation and periodate oxidation evidence for the presence of 1,3- linked galactose, 1,3- linked arabinose and 1,4- linked xylose. These features have been confirmed in this latest investigation, again by periodate oxidation. Moreover, evidence has been obtained, from partial acid hydrolysis studies, for the presence of 1,3- and 1,6- linked galactose, 6-sulphated galactose and 1,4- or 1,5- linked 3-sulphated arabinose units. Evidence has also been presented that the related genus, Chaetomorpha, synthesises comparable sulphated polysaccharides.

It was hoped that, by application of the Smith degradation procedure, some information might be obtained about the basic core of the Cladophora polysaccharide molecule. The only conclusion which could be drawn, however, was that this

consisted of galactose and arabinose, probably combined together in a single heteropolysaccharide.

It is interesting that, although the polysaccharides of Cladophora and Codium have some features in common, such as 6-sulphated and 1,3- linked galactose, they are apparently quite dissimilar substances. For example, although each polymer contains alkali-labile ester sulphate, this is mainly situated on arabinose units in Cladophora, and on galactose units in the Codium polysaccharide. This fundamental structural difference is strikingly emphasised when the polysaccharides are treated with sodium methoxide. Major proportions of monomethyl pentose are incorporated into the Cladophora polysaccharide, whereas the Codium polymer appears to be virtually unaffected.

Fractionation studies of Cladophora polysaccharide have allowed the separation of a purified galactose-arabinose-xylose polymer, and at the same time, suggested that other minor polysaccharide components are present in the original extract. It is still not certain, however, that this main fraction is truly a single polysaccharide. Although the balance of evidence from the previous and the present studies suggests that this is in fact so, all attempts to verify this by the isolation of hetero-oligosaccharides from fragmentation studies were unsuccessful. This problem is a general one in green seaweed sulphated polysaccharides, and is probably the most outstanding unsolved feature of these materials.

Until unequivocal evidence is obtained to the contrary,

it therefore appears that the sulphated polysaccharide of Cladophora (and probably Chaetomorpha) is best regarded as a family of related, polydisperse molecules, in the same way as the sulphated polysaccharides of red algae, and the uronic acid containing, sulphated polymers of some green algae.

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W J Mackie

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790. *Starch-type Polysaccharides Isolated from the Green Seaweeds,* Enteromorpha compressa, Ulva lactuca, Cladophora rupestris, Codium fragile, *and* Chaetomorpha capillaris.

By J. Love, W. Mackie, J. W. McKinnell, and Elizabeth Percival.

Starch-type polysaccharides have been isolated, and fractionated into an amylose and an amylopectin component, from four genera of green seaweed. The conditions of isolation from *Chaetomorpha* were such as to destroy any amylose, and only the amylopectin of this genus was separated and characterised. Although apparently considerably smaller molecules, the algal amyloses and amylopectins resembled potato amylose and amylopectin, respectively, in many of their properties.

The only reported isolations of characterised starch-type polysaccharides from green seaweeds are those of an amylopectin-type polymer from *Caulerpa filiformis* [1] and a starch-type polymer from *Codium fragile*.[2] The former of these was fractionated with cetyltrimethylammonium bromide from the water-soluble polysaccharides, and the latter was precipitated as the starch–iodine complex from the hot-water extract.[3] We have now succeeded in fractionating starch-type polymers as the starch–iodine complex from the water-soluble polysaccharides of *Enteromorpha compressa*, *Ulva lactuca*, and *Chaetomorpha capillaris*. Owing to gelling of *Cladophora rupestris* polysaccharide, it was advantageous to separate the starch from the aqueous extracts before isolation of the sulphated polysaccharides and *ca.* 50-g. batches of dry weed gave the highest percentage yield of starch. The same procedure was also used for *Codium fragile*.[2] The yields of the respective starches based on the dry weight of weed are: *C. fragile*, *ca.* 1·0%; *E. compressa*, 0·6%; *U. lactuca*, 1·2%; *C. capillaris*, 0·3%; *C. rupestric*, 1·0%. An acid hydrolysate of each product contained glucose as the sole carbohydrate (paper chromatography and treatment with glucose oxidase). The glucose contents were determined by cuprimetric titration, and all the analytical figures of the different fractions are based on their glucose content. Most of the starches were contaminated with ash (0·9—2·0%) and protein (up to 5%).

Apart from the material from *C. capillaris*, which gave a purple colour, all the products gave a blue colour with iodine and were fractionated by the thymol method, and the resulting amylose complexes were purified by two precipitations with butanol (for percentage of amylose see Table 1). In each fractionation a proportion of material was lost, probably during dialysis of the solution containing the amylopectin.

The intrinsic viscosity, blue value (B.V.),[4] and $\lambda_{max.}$ (mμ) (wavelength of peak absorption of iodine complex) for each of the amyloses and for potato amylose measured under identical conditions are given in Table 1, and those of the respective amylopectins in Table 2. An estimate of the degree of polymerisation ($\overline{DP}$) of the amyloses was made from the approximate relation, $\overline{DP} = 7{\cdot}4[\eta]$ [5] (see Table 1).

The quantity of periodate reduced [6] corresponds to *ca.* 1 mole for every anhydroglucose unit, for each of the amyloses (see Table 1) and amylopectins (Table 2), and the oxopolysaccharides were devoid of unattacked glucose units. This provides qualitative evidence that 1,2- and 1,3-glucosidic linkages are absent and that the polysaccharides contain 1,4-linked glucose units with possible branch points at C-6. Confirmation of this structure was obtained for the amylopectins from *E. compressa*, *C. rupestris*, and *C. fragile*, from methylation studies.

Parallel experiments with β- and α-amylase on each of the algal glucans and on potato amylose and amylopectin were carried out. The apparent percentage conversion into maltose (P_M) was measured and the results are given in Tables 1 and 2. The β-amylolysis limits with a Wallerstein β-amylase preparation, which was known to be contaminated

with *Z*-enzyme, were measured at pH 3·6 and 4·6. At pH 3·6 the action of *Z*-enzyme is inhibited.[7] In the present experiments the action of the contaminating *Z*-enzyme is so slight that only a minute proportion of the α-1,4-glucosidic linkages in amylopectin are cleaved, so that the resultant increase in β-amylolysis limit is very small. On the other hand, amyloses are essentially linear and slight *Z*-enzyme activity in the case of potato amylose was sufficient to result in complete fission to maltose. Considerable difficulty was encountered in carrying out the amylolyses at pH 3·6 with the algal amyloses which readily retrograde from solution. Additional experiments with a purified β-amylase which was free from *Z*-enzyme and maltase activity were carried out on the amylopectins of *C. rupestris*, *U. lactuca*, *C. fragile*, and potato, glutathione being used as an activator. Since glutathione interferes with cuprimetric estimation, the maltose concentrations were measured by ferricyanide–ceric sulphate titration.[5] Addition of *Z*-enzyme to the amylopectin digests increased the β-amylolysis limits to a small extent (see Table 3).

TABLE 1.

Properties of amylose-type molecules.

	Cladophora rupestris	*Enteromorpha compressa*	*Ulva lactuca*	*Codium fragile*	Potato
% Amylose in Starch	20	22	37	16	25
$[\eta]$	78	44	41	36	300
$\overline{DP}$	577	325	302	266	2220
Blue value	1·2	0·66	1·14	0·96	1·21
$\lambda_{max.}$	635	610	630	610	640
$[\alpha]_D$	+158° *	+177°	+161° *	+197°	+157° *
β-Amylolysis: pH 3·6, P_M	73	—	71	84	80
pH 4·6, P_M	88	76	90	96	100
Reduction IO_4^-/anhydro-unit	0·97	1·08	1·01	1·10	1·01

* Measured in molar KOH.

TABLE 2.

Properties of amylopectin-type molecules.

	Cladophora rupestris	*Enteromorpha compressa*	*Ulva lactuca*	*Codium fragile*	*Chaeto-morpha*	Potato	Glycogen
$[\eta]$	41	35	58	24	—	161	10
Blue value	0·196	0·140	0·220	0·220	0·108	0·176	—
$\lambda_{max.}$	565	550	560	560	540	560	460
$[\alpha]_D$	+197°	+190°	+205°	+192°	—	+197°	+196°
α-Amylolysis, P_M	92	90	91	84	81	92	70
β-Amylolysis: pH 3·6, P_M	55	—	[redacted]	—	—	53	
pH 4·6, P_M	57	58	56	—	62	56	45
Reduction IO_4^-/anhydro-unit	1·05	0·900	1·05	1·2	1·11	1·04	1·0

TABLE 3.

	β-Amylolysis			β-Amylolysis	
Amylopectin	β-Limit P_M	β + *Z*-Limit P_M	Amylopectin	β-Limit P_M	β + *Z*-Limit P_M
C. rupestris	50	55	*C. fragile*	51	53
U. lactuca	51	55	Potato	53	57

The present study has shown that four different genera of green seaweed synthesise a starch which can be fractionated into essentially linear (amylose) and branched (amylopectin) components. Apart from that in *U. lactuca* which is higher (37%), the proportion of amylose (16—22%) in the other genera is comparable with that of the majority of plant starches (20—30%), although certain varieties of pea [8] and maize [9] starches have a much higher amylose content. The algal amyloses have a low iodine-binding power and this may be correlated with their low $\overline{DP}$, since there is evidence that $\lambda_{max.}$ of amylose-type chains is directly related to the $\overline{DP}$ [10] if this is lower than 500. Amylose is readily degraded by oxygen and alkali, and for this reason the present starches were extracted and fractionated under the mildest possible anaerobic conditions. It seems very probable,

therefore, that they are smaller molecules than plant amyloses. Microscopic examination certainly indicates a less organised granule. The difficulty of obtaining large weights of *C. capillaris* precluded separate extraction of the starch and the sulphated polysaccharide, and consequently the method used, which gave a relatively pure yield of the latter (boiling water in contact with air and then treatment overnight with 4% trichloroacetic acid) was sufficiently drastic to destroy any amylose present in the native starch and it was only possible to isolate and examine an amylopectin component. That the native *Chaetomorpha* contained an amylose component was deduced from the deep blue colour given with iodine by solutions obtained by extraction of the weed for 15—20 minutes with ~~cold~~ hot water; successive samples taken during prolonged extraction with boiling water showed a progressive change in the colour given with iodine to the typical purple colour produced with amylopectins. Similar hot-water extractions of *C. rupestris* in contact with air also produced a polysaccharide mixture which gave a purple rather than the blue colour with iodine solution.

The high positive rotation of each of the amyloses, their ready retrogradation from aqueous solution, and the reduction by them of *ca.* 1 mole of periodate for every anhydroglucose unit all confirm their essentially linear α-1,4-linked structure. The β-amylolysis limits, apart from those of *E. compressa* amylose, are only slightly lower than that of potato amylose and are further evidence of their similarity with the latter.

The algal amylopectins apart from their low intrinsic viscosities show even more striking similarity with potato amylopectin. Their rotations, $\lambda_{max.}$, and α- and β-amylolysis limits are the same as those of potato amylopectin and, like the latter, they reduce *ca* 1 mole of periodate for every anhydroglucose unit. The hydrolysate from the methylated amylopectin from *E. compressa* contained tetra- to tri-*O*-methylglucose in the ratio of 1 : 26. This indicates an average chain-length for *E. compressa* amylopectin of *ca.* 27, a value slightly higher than that of the majority of plant amylopectins whose average chain-length is *ca.* 25. The tetra- and tri-*O*-methylglucoses from methylated *Cladophora* amylopectin were separated and characterised as crystalline sugars.

Experimental

The analytical methods used have been described by O'Donnell and Percival.[11] Blue values refer to 680 mμ throughout.

Isolation of the Starches.—Finely powdered *C. rupestris* weed (30—40 g. batches) was freed from colouring matter by immersion in butanol half-saturated with water overnight, followed by several extractions with cold acetone. The decolorised weed in water (500 ml.) was thoroughly saturated with nitrogen and the mixture heated to 90—95° with continuous stirring and passage of nitrogen. After 1 hr. the mixture was cooled and the residual weed removed by centrifugation. The residue, after being washed with warm water, was re-extracted as before. The extract and washings were concentrated to 200 ml. and the starch-type polysaccharide was separated from this solution as the starch–iodine complex.[3] The Celite was reduced to 1·5 g. for each 40 g. of weed extracted. Precipitation of the starch–iodine complex was repeated before removal of the Celite, and the combined precipitate and Celite were then suspended in 95% ethanol (200 ml.) before dropwise addition of thiosulphate solution. When the blue colour had disappeared, the ethanol concentration was reduced to 70% with water and the starch–Celite mixture filtered off and washed with 70% ethanol (200 ml.). The starch–Celite was made into a paste with water and plunged into water (100 ml.) at 90° in an atmosphere of nitrogen. After vigorous stirring for 15 min. the Celite was removed by high-speed centrifugation. The supernatant solution was dialysed before freeze-drying. The yield of a white amorphous powder was 0·4 g. from 40 g. of weed.

The starch-type polysaccharides from the other genera were separated, essentially as above,[3] from aqueous solutions of the isolated water-soluble polysaccharides. An acid hydrolysate of each of the products contained glucose only (paper chromatography and glucose oxidase treatment).

Separation of Amylose and Amylopectin from the Starch-type Polysaccharides.—The starch (800 mg.), made into a paste with water, was plunged with vigorous stirring into water (100 ml.)

at 95° in an atmosphere of nitrogen. After 20 min. the solution was allowed to cool to 60° and then saturated with powdered thymol (0·13 g.). The mixture was stirred for 30 min. at 55—60° and finally allowed to cool to room temperature with constant stirring and in an atmosphere of nitrogen throughout. After 2 days a copious white thymol–polysaccharide precipitate had been deposited. This was removed on a high-speed centrifuge and dispersed with stirring under nitrogen in distilled water at 90°. The centrifugate was freed from thymol by extraction with ether, and amylopectin (400 mg. from *C. rupestris*) was isolated from the aqueous solution by freeze-drying after dialysis. It gave a purple colour with iodine and had $[\alpha]_D$ +197° (*c* 0·4) [Found: glucose by cuprimetric titration [12] of a hydrolysate (2N-sulphuric acid at 100° for 2·5 hr.), 81%; ash, 2·0%; protein-N, 0·8%]. Amylopectins from *E. compressa* (Found: glucose, 91%; ash, 0·9%; protein-N, nil), *Ulva lactuca* (Found: glucose, 86%; ash, 1·0%; protein-N, 0·4%, and *C. fragile* (Found: glucose 81%; whole starch ash, 1·8%; protein-N, 0·8%), were similarly obtained. Compare amylopectin (Found: glucose 95%); potato (Found: glucose, 95%).

The dispersed amylose solution was saturated with butanol (10%) at 60°, the mixture allowed to cool to room temperature with stirring, then stoppered and set aside overnight. The deposited butanol–amylose complex was removed at the centrifuge and recrystallised again with butanol. The derived amylose (160 mg. from *C. rupestris*) gave a blue colour with iodine and had $[\alpha]_D$ +158° (*c* 0·7 in N-NaOH). The starch from *U. lactuca* (400 mg.), which was insoluble in water, was dissolved in 0·5N-sodium hydroxide with stirring under nitrogen. The alkaline solution was dialysed against running water until neutral (6 days) and then concentrated at 40° to 100 ml. before fractionation with thymol. By cuprometric titration as for the amylopectins the percentage glucose contents of the amyloses were found to be *C. rupestris* 83, *E. compressa* 80, *C. fragile* 95.

Viscosity Determination.[5]—The specific viscosity (η_{sp}) of N-potassium hydroxide solutions of the polysaccharides (0·5—1·5%) saturated with nitrogen were determined at several concentrations at 25° in a Ubbelohde viscometer and the limiting viscosity numbers determined (see Tables 1 and 2).

Enzymic Degradation.—(*a*) *Salivary α-amylase.* Algal amylopectin and potato amylopectin solutions (0·05—0·1%) in 0·04M-citrate–phosphate buffer (pH 7·0), sodium chloride (1 mg.), and purified salivary α-amylase [13] (0·1%) in a total volume of 20 ml. each were incubated separately at 37° for 48 hr. and analysed for their maltose content by cuprimetric titration.[12] The P_M values are given in Table 2.

(*b*) *β-Amylase.* (i) Digests of the respective algal amyloses, amylopectins, and potato amylose and amylopectin solutions (0·05—0·1%), were prepared in acetate buffer (pH 4·6; 0·04M), serum albumin (0·01—0·05%), and Wallerstein β-amylase preparation (0·05—0·1%; 110 units/mg. assayed by the method of Hobson, Peat, and Whelan [14]), in a total volume of 20—25 ml. and incubated at 37°. The P_M values are given in Tables 1 and 2.

(ii) Similar digests were prepared of *C. rupestris*, *U. lactuca*, *C. fragile*, and potato amylopectins, except that purified β-amylase (3000 units/mg.[14]) free from *Z*-enzyme and maltase activity in 0·5M-glutathione was used. The P_M was determined after ~~a further~~ 24 hr.[5] (see Tables 1 and 2). then Wallerstein enzyme (5 mg.) added and PM determined after a further 24 hrs.

Periodate Oxidation.—Polysaccharide samples (10 mg.) were oxidised with aqueous 0·015M-sodium metaperiodate (25 ml.) in the dark at 4°. Aliquot parts (0·1 ml.) were analysed at intervals for the amount of periodate reduced.[6] Chromatographic examination of a hydrolysate of the reduced ($NaBH_4$) oxo-polysaccharides after oxidation for 98 hr. showed the absence of glucose.

Periodate oxidation of the starch fractions. Moles IO_4^- reduced/anhydro-unit.

Time (hr.)	20	44	68	98
Potato amylose	0·684	0·891	0·991	1·01
amylopectin	0·844	0·964	1·03	1·04
C. rupestris amylose	0·488	0·699	0·90	0·97
amylopectin	0·840	0·890	1·02	1·05
E. compressa amylose	0·800	0·890	0·95	1·08
amylopectin	0·70	0·79	0·81	0·90
U. lactuca amylose	0·424	0·748	0·94	1·01
amylopectin	0·700	0·996	1·03	1·05
C. fragile amylose	0·84	0·95	1·00	1·10
amylopectin	1·02	1·19	1·21	1·21

Methylation of the Amylopectin Fractions.—(a) *From* C. fragile. Amylopectin (120 mg.) in water (10 ml.) was treated overnight at room temperature with potassium borohydride (50 mg.). 60% Sodium hydroxide solution (10 ml.) and dimethyl sulphate (6 ml.) were then added dropwise during 6 hr. Throughout these additions the mixture, saturated with nitrogen, was stirred vigorously and kept below 2°. It was then stirred at room temperature overnight. The methylation was repeated twice under exactly the same conditions. The partially methylated product (100 mg.) was isolated by freeze-drying after dialysis. The above methylation procedure was repeated thrice more and the polysaccharide isolated after each methylation as before. The product was extracted overnight with chloroform under reflux and the chloroform-soluble material dried overnight at 60° *in vacuo*. It (*ca.* 30 mg.) was subjected to two Purdie methylations with methyl iodide (10 ml.) and dry silver oxide (2·0 g.) added during 5 hr.

(*b*) *From* E. compressa. The amylopectin (52 mg.) was mixed with Filter-Cel and methylated with potassium and methyl iodide in liquid ammonia.[15] After four complete methylation cycles the products were extracted with dry boiling chloroform (20 ml. × 5) for 2 hr.

(*c*) *From* C. rupestris. The amylopectin (100 mg.) was subjected to three complete methylations as under (*b*), and the product isolated from the chloroform solution as a pale yellow mobile syrup (105 mg.).

Hydrolyses of the Methylated Polysaccharides.—(i) The methylated amylopectins (*ca.* 15 mg.) were refluxed separately with 2% methanolic hydrogen chloride for 8 hr. After neutralisation with silver carbonate, filtration, and concentration, the methyl glucosides were examined by gas chromatography.[16] Methyl 2,3,4,6-tetra-*O*-methyl-α- and -β-glucoside, methyl 2,3,6-tri-*O*-methyl-α- and -β- glucoside, and two small peaks characteristic of methyl di-*O*-methylglucosides were detected.

(ii) The methylated glucosides from *E. compressa* were hydrolysed by N-sulphuric acid at 100° for 6 hr., and the derived syrups separated on Whatman No. 1 paper eluted with ethyl methyl ketone saturated with water containing 0·1% of ammonia. The paper was dried after elution for 3 hr. and then eluted with the same solvent for a further 6 hr. The portions of paper containing the tetra- and tri-*O*-methyl sugars, together with comparable areas of blank paper, were eluted with water and methanol. The eluates, after concentration to dryness, were made up to a standard volume with water, and the sugar contents of aliquot parts were determined [17] after the preparation of standard graphs. Values given by the blanks were subtracted from the respective sugars. The ratio of tetra- to tri-*O*-methylglucose was 1 : 26.

(iii) The methylated amylopectin from *C. rupestris* (72 mg.) was hydrolysed with 90% formic acid (4 ml.) in an atmosphere of carbon dioxide in a sealed tube at 100° for 6 hr. The solution was cooled and neutralised with Amberlite IR-4B(OH^-) resin, and a portion of the hydrolysate (60·0 mg.) was separated on Whatman 3 MM paper in solvent 6.[11]

Fraction I was crystalline 2,3,4,6-tetra-*O*-methylglucose (5·0 mg.), chromatographically and ionophoretically identical with an authentic specimen run as a control. It had m. p. and mixed m. p. 84°; the derived anilide had m. p. 137°, undepressed on admixture with authentic material.

Fraction II was crystalline 2,3,6-tri-*O*-methylglucose (50 mg.), m. p. and mixed m. p. 115° (from ether), $[\alpha]_D$ +68° (*c* 1·5). The derived diethyl mercaptal had m. p. 70°.[18]

Fraction III was syrupy di-*O*-methylglucose which was not characterised.

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spots of acid glycosaminoglycan solution on filter paper, stained with 0·1% aqueous Alcian Blue 8GX, in 0·05 M-sodium acetate buffer (pH 5·7) containing NaCl, $MgCl_2$ or $AlCl_3$, showed that Alcian Blue behaves similarly as cetylpyridinium, e.g. hyaluronate stains at $MgCl_2$ concentrations < 0·1 M, DNA < 0·25 M, chondroitin sulphuric acid < 0·5 M, heparin < 0·6 M, keratosulphate < 0·8 M.

Histological sections of the hilar region of human newborn lung, guinea pig cornea, rat ear and ileum showed that in general, mucins and –COOH containing glycosaminoglycans took up stain at < 0·3 M $MgCl_2$, nuclei at < 0·2 M, chondroitin sulphuric acid in hyaline cartilage at < 0·7 M, mast cells (containing heparin) < 0·9 M, and cornea (containing keratosulphate) took up stain even at 1·0 M. This system provides an exceptionally clear way of demonstrating mast cells. The critical electrolyte concentration at which staining was suppressed was higher by 50% in sections than with model experiments on paper.

Heath (1961) showed that sulphated polysaccharides were the only substrates to take up certain dyes in solutions of aluminium salts, and Saunders (1962) demonstrated a critical electrolyte concentration differentiation of acid glycosaminoglycans using acridine orange, based on its supposed similarity to cetyl pyridinium with regard to micelle formation. We suggest that differentiation based on critical electrolyte concentration is a quite general possibility. The activity of the precipitating cation is maintained at the same level throughout the series of salt solutions, e.g. by having excess solid phase, or non-reactive micelles present, or by having sufficient excess of precipitant so that the effective concentration does not decrease during the precipitating (staining) reaction, as in our experiments.

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(2)

The Site of Ester Sulphate Groups in the Polysaccharide from the Green Seaweed, *Cladophora rupestris*

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The water-soluble sulphated polysaccharide from *Cladophora rupestris* (Fisher & Percival, 1957) after separation of a starch, comprises L-arabinose, D-galactose and D-xylose with small amounts of D-glucose and L-rhamnose.

Partial hydrolysis of a 2·5% solution of the polysaccharide (10 g.) with *N*-sulphuric acid for 1·5 hr. at 100° gave a syrupy mixture of neutral and acidic mono- and oligo-saccharides. Chromatographic separation of this syrup sequentially on Amberlite IR 400 A (acetate form) resins, charcoal, and Whatman 3MM paper gave two pure acidic materials, isolated as their ammonium salts: Fraction 1 (95 mg.) with $R_{\text{galactose 6-sulphate}}$ 1·0; $R_{\text{galactose}}$ 0·55 (ethylacetate/pyridine/water, 10:4:3, by vol.); $M_{\text{galactose 6-sulphate}}$ 1·0 (electrophoresis in pyridine–acetic acid buffer, pH 6·0; 0·05 M), $[\alpha]_D$ + [illegible]° (*c* [illegible], H_2O). It gave galactose on hydrolysis, had a DP of 1·02 (Timell, 1960) and a galactose to sulphate (Jones & Letham, 1954) molar ratio of 1:1. Gas–liquid chromatography (Aspinall, 1963) of a methanolysed, methylated (Kuhn, 1955) aliquot gave peaks with the retention times of methyl 2,3,4-tri-*O*-methylgalactosides. Further proof that fraction 1 is galactose 6-sulphate was obtained by oxidation of an aliquot with 0·015 M-sodium periodate (Aspinall & Ferrier, 1957). Following an initial rapid reduction of 3 moles, oxidation was complete after 96 hr. with the reduction of 3·79 moles of periodate/mole of galactose 6-sulphate (theory requires 4 moles) and no liberation of formaldehyde (cf. Turvey, Clancy & Williams, 1961).

Fraction 2 (56·5 mg.) had $R_{\text{galactose}}$ 0·83, $M_{\text{galactose 6-sulphate}}$ 1·18, $[\alpha]_D$ + 75° (*c* 0·60, H_2O). It gave arabinose on hydrolysis, had a DP of 1·03 and a molar ratio of arabinose to sulphate of 1:1. Gas–liquid chromatography of a methanolysed methylated aliquot gave peaks with the retention times of methyl 2,5- and 2,4-di-*O*-methylarabinosides. Confirmation that fraction 2 is arabinose 3-sulphate was obtained by the reduction of 2·95 moles of periodate and the release of 0·48 mole and 0·79 mole of formaldehyde/mole arabinose sulphate (MacFadyen, 1945) in unbuffered and in bicarbonate-buffered (pH 7·5) solution respectively. In parallel experiments, glucose 3-sulphate released 0·38 and 0·81 mole of formaldehyde/mole (theory requires the reduction of 3 moles of periodate and the liberation of 1 mole of formaldehyde from arabinose 3-sulphate). Controlled hydrolysis studies (Rees, 1963) on fractions 1 and 2 have a half life of 1·61 and 0·80 hr. respectively.

The presence of 3-sulphated arabinose in the polysaccharide was confirmed by the formation of 2-*O*-methyl-L-xylose on treatment of the polymer with sodium methoxide. This is the first sulphated arabinose to be found in nature.

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